CARBON AND NITROGEN FLOWS IN ZERO-WATER EXCHANGE SHRIMP CULTURE: INFERENCES USING STABLE ISOTOPE TRACERS

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Michelle A. Epp, B.A., M.S.

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Michelle A. Epp

RECOMMENDED:

Advisory Committee Chair

Program Head

APPROVED:

Dean, School of Fisheries and Ocean Sciences

Dean of the Graduate School

8-9-02

Date

ABSTRACT

Nutrient and energy flow in cultures of Pacific White Shrimp, *Litopenaeus vannamei*, were examined in zero-water exchange, 1200 - 1300 L mesocosms at the Oceanic Institute (OI), Waimanalo, Hawaii. A technique was developed for monitoring shrimp use of formulated feeds through the addition of stable isotopically labeled nutrients to the feed ingredients. Crystalline amino acid compounds were ineffective as labels due to their rapid dissolution in the tank water with feed pellet break-up. Labels which were 'packaged' as algal cells prior to addition to the feed pellets were more effectively incorporated into shrimp tissues than crystalline label (approximately 27% versus 8% for crystalline label). The 'packaged' label technique was also used to test soluble proteins from pollock processing wastes (stickwater) as a feeding stimulant for *Litopenaeus vannamei*. Indoor controlled condition experiments and outdoor experiments with natural pond biota compared stickwater amended feed to squid liver powder amended feed for growth and assimilation by the shrimp. Initial results indicated that pollock processing by-products might function as a feeding stimulant in shrimp aquaculture.

The addition of ^{15}N-ammonium to outdoor shrimp tanks showed that natural tank production contributed significantly to shrimp growth requirements providing

between 17 and 77% of the growth nitrogen. When labeled ammonium was added to black covered tanks, shrimp had slower growth rates (0.5 g/wk as compared to 0.7 g/wk for uncovered ammonium addition tanks) but significant uptake of this label, with a tank biota contributing 23%. This finding supported a bacterial role in shrimp nutrition that will require further study. Isotopic analysis of individual amino acids in shrimp muscle from outdoor tanks with and without added ^{15}N-ammonium further established the role of tank natural populations to shrimp nutrition. Rapid increases in $\delta^{15}N$ for threonine one day after label addition suggested an increased requirement for this essential amino acid. Further identification of the contribution of tank biota to shrimp amino acid profiles will require profiles of the $\delta^{15}N$ of specific amino acids for suspended particulate organic matter.

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CHAPTER 1 – BACKGROUND

The United States is one of the leading importers of shrimp in the world (FAO, 1998). Commercial shrimp landings in the U.S. totaled 277.8 million pounds in 1998, whereas imports were more than double landing totals at 695.4 million pounds (NMFS, 1999). The U.S. imported a record 728 million pounds in 1999 (NMFS, 2001), and 760 million pounds in 2000 (NMFS, 2001). Thus, the contribution to the trade deficit from shrimp products has increased over the last few years, from $3.1 to $3.8 billion/year (NMFS, 2001).

It is evident that the United States needs to explore its options for decreasing the trade deficit and to improve domestic shrimp production. This is particularly important in light of recent concerns about overfishing wild stocks. Shrimp catches declined 20% in the U.S. from 1989 to 1998 while imports increased 59% (NMFS, 1999). Furthermore, bycatch resulting from shrimp fishing is also a concern. The National Audubon Society noted that for every pound of shrimp caught, 7 pounds of bycatch were discarded (National Audubon Society, 1998). This fact contributed to their warning that shrimp consumption has a dangerous impact on the marine environment (National Audubon Society, 1998).

Domestic shrimp aquaculture may be instrumental in ameliorating overfishing and in lowering the trade deficit. In the countries from which the U.S. imports most of its shrimp (Thailand, 29% and Ecuador, 20%), shrimp farming provides a sizeable portion of their exports (69% and 90% respectively, (NMFS, 1999)). The United States, however, has experienced a general decline in aquaculture production of marine shrimp from 1993 to 1997. Aquaculture in the U.S. provided only 2.6 million pounds of marine shrimp in 1997 (NMFS, 1999). In addition to limiting the overall trade deficit, aquaculture can contribute significantly to regional economies. In Hawaii, shellfish production from aquaculture has increased from a value of $4.3 million in 1996 to $7.7 million in 2000 (HASS, 2001).

Shrimp aquaculture systems are generally divided into 4 groups: extensive, semi-intensive, intensive, and ultra- or super-intensive (Fast, 1992; Lee and Wickins, 1992). Extensive systems were the first form of shrimp farming. Wild stocks of shrimp are caught and maintained in large (> 5 ha) penned-off areas along the coast (Fast, 1992). Tidal exchange flushes the pond, and natural phytoplankton and secondary production within the pond provides the food source for the shrimp (Lee and Wickins, 1992). Shrimp are stocked at low densities (0.1 to 5 shrimp m^{-2}) in these systems, and the yield is also low (< 1 mt ha^{-1}, Fast, 1992; Lee and Wickins, 1992).

Shrimp farming has been intensified, with increased demand for shrimp products, using smaller ponds and higher stocking densities of cultured rather than wild postlarvae (Landesman, 1994). In semi-intensive systems, pumps supplement water exchange for aeration in 3 to 20 ha ponds, and formulated feeds are provided to supplement pond natural production. Production increases to 5 mt ha^{-1} with stocking densities of 10 to 20 shrimp m^{-2} (Fast, 1992; Lee and Wickins, 1992). Aeration in intensive and ultra-intensive systems must be provided exclusively by pumps, and shrimp nutrition is primarily from formulated feeds. Water exchange rates of 10 to 20%/day for intensive systems and more than 100%/day in ultra-intensive systems are required to address nutrient build-up and waste within the ponds (Fast, 1992). Pond sizes in intensive and ultra-intensive systems decrease to 1 ha and < 0.25 ha while production increases to 5 - 15 mt ha^{-1} and > 30 mt ha^{-1} respectively (Fast, 1992; Lee and Wickins, 1992).

Effluent is a major concern given the high densities and reliance on formulated feeds (Schwartz and Boyd, 1992). Waste in aquaculture systems is generally high in dissolved nutrients which, when released to the natural waters, can result in nuisance algal blooms (Boyd, 1999). In some semi-intensive systems, effluent causes 'self-pollution' via partial recycling of water (Rivera-Monroy et al., 1999) as well as

problems for the surrounding ecosystems. The tidal flow that removes toxic levels of ammonia from one pond can transport it to nearby ponds with potentially harmful effects (Ziemann et al., 1992). The National Audubon Society also noted this as part of their warning. While some members of the aquaculture community argue that this presents only part of the story (Chamberlain, 1999), increased efforts to tighten legislation pertaining to aquaculture effluent mean that advances in the field will require addressing this issue (Schwartz and Boyd, 1992). Effluents markedly increase the operating costs due to processing of wastes, and a limit to the cost-effectiveness of shrimp production in the U.S., particularly when the industry has to compete with imports from countries with low wages, less stringent laws governing discharges, and larger allotments of land for shrimp aquaculture use.

An important step in reducing the amount of wastes produced in shrimp culture tanks is to provide nutritionally complete feed (Boyd, 1999). Shrimp are inefficient feeders that leave behind broken feed pellets to decompose and mineralize within the system, thus increasing the amount of potentially harmful nutrients in the pond (Lee and Wickins, 1992). In addition to effluent concerns, feed formulations can account for 25-50% of the cost of shrimp rearing in intensive and semi-intensive systems (Tacon,

1999). Therefore, an understanding of the nutritional ingredients required by the shrimp will not only aid in better feed conversion, but will reduce feed costs.

The usual technique for determining the necessary ingredients in shrimp diets is to compare growth response on feeds with and without a given component. This type of study must be performed under controlled conditions in order to limit the number of confounding variables that are present in outdoor systems. Under normal culture conditions, shrimp nutrition is supplemented with natural primary and secondary pond production, thereby making laboratory studies less applicable to natural systems (Anderson et al., 1987; Nunes et al., 1997; Parker et al., 1989). While gut content analysis can give some insight into what the shrimp are consuming (Focken et al., 1998), it is not necessarily representative of the assimilated carbon and nitrogen sources (Dittel et al., 1997).

Stable isotope analysis can often provide a clearer view of those sources. Stable isotopes are useful tracers in studying food web dynamics because they are naturally occurring and can be determined with a high degree of accuracy (see review by Wada et al., 1991). Their usefulness derives from the fact that each isotope behaves essentially identically in a chemical sense, and thus the isotope ratios are conservative tracers in the transfer of food to organism. By comparing the isotopic ratio in the organism of interest

to the ratio in possible food sources, one can make inferences about what the organism is eating.

The benefits of isotope analysis of food webs in shrimp aquaculture were first described by Schroeder (Schroeder et al., 1984; Schroeder, 1983a; Schroeder, 1983b) for penaeid species and *Macrobrachium rosenbergii*. Stable isotopes have been used repeatedly since that time to examine food sources for shrimp (Anderson et al., 1987; Nunes et al., 1997; Parker et al., 1991). Most studies have measured the naturally occurring isotope ratios of carbon and nitrogen (natural abundance). Small variations in isotope ratios occur naturally and, if food sources are sufficiently different, these can be useful in allocating energy or nutrient sources. There are, however, limitations to natural abundance studies. The isotopic values for the food sources have to be known precisely, and minor sources with different isotope ratios can bias the results. These difficulties can be overcome by deliberately changing the ratio of the heavy to light isotopes for one of the feed components. By subsequently following the change in stable isotopic ratios in the shrimp over time, it is possible to allocate the amount of carbon and nitrogen derived from labeled feed, versus that incorporated from pond biota, and gain information on nutrient pathways under normal culture conditions.

The overall objective of this work was to determine the energy and nutrient flows within culture tanks of *Litopenaeus vannamei*, Pacific white shrimp. These experiments were performed in zero-water exchange systems at the Oceanic Institute (OI), Waimanalo, Hawaii. This type of shrimp management has minimal effluent and will reduce the negative impacts to the surrounding environment (Boyd, 1999). The results of this study are expected to improve shrimp culture through advances in feed formulations. The specific objectives will be discussed in the following chapters and were addressed during 4 experimental periods (Table 1.1).

Chapter 2 examines the role of formulated feeds in shrimp growth. Methodology was developed to label feeds with stable isotopes for shrimp aquaculture studies. The incorporation of feeds, labeled with amino acids or organically 'packaged' labeled compounds (isotopically labeled algal cells), into shrimp muscle and carapace tissues was compared.

Chapter 3 discusses the use of the developed methods to determine whether fishery by-products, specifically pollock stickwater, can be used as an effective feed attractant. The Alaskan fishery industry produces about 1 million tons of by-products a year, which are either discarded or further processed to make secondary products such as fishmeal. Efforts are underway to find economically viable products from these

Table 1.1: Overview of *Litopenaeus vannamei* feeding experiments

OML #	Trial Dates	Stocking Density (#/tank)	Trials
98-1	17 March - 5 May 1998	90	Controls ^{13}C-glycine labeled feed ^{15}N-glycine labeled feed ^{15}N-amino acid mix labeled feed $^{15}NH_4Cl$ addition to tanks
98-5	16 September - 9 December 1998	90	Controls ^{15}N-glycine labeled feed ^{15}N-amino acid mix labeled feed $^{15}N/^{13}C$- algal cell labeled feed $^{15}NH_4Cl$ addition to tanks
99-3	20 July - 14 September 1999	100	*Covered and Uncovered Tanks* Controls $^{15}N/^{13}C$- algal cell labeled feed $^{15}NH_4Cl$ addition to tanks ^{13}C-mannitol addition to tanks
01-1	4 January - 1 February 2001	100	Controls ^{15}N-algal cell labeled 'green' (plant based) feed ^{15}N-algal cell labeled green feed minus squid liver powder (standard attractant) ^{15}N-algal cell labeled green feed + 1% Stickwater (pollock processing waste) ^{15}N-algal cell labeled green feed + 2.5% Stickwater $^{15}NH_4Cl$ addition to tanks $^{15}NH_4Cl$ addition to black covered tanks
ICL 01-2	5 January - 2 February 2001	36	^{15}N-algal cell labeled green feed ^{15}N-algal cell labeled green feed minus SLP ^{15}N-algal cell labeled green feed + 1% Stickwater ^{15}N-algal cell labeled green feed + 2.5% Stickwater ^{15}N-algal cell labeled indoor control feed

materials in order to increase recycling and limit waste. Stickwater is produced during fish processing and meal production. It consists primarily of soluble and colloidal fish derived proteins in aqueous solution. This study used only stickwater produced in plants using freshwater clean-up, as seawater wash contains excessive salt for economic drying. Given its composition, stickwater may prove useful as an attractant.

Chapter 4 investigates of the role of natural tank assemblages in shrimp growth. As indicated above, shrimp also utilize natural pond production as a normal part of their diet. As shrimp rearing conditions intensify, their reliance on natural food sources is diminished with increased reliance on the addition of formulated feeds. However, shrimp that are reared in the presence of both formulated feeds and natural production do better than those reared on formulated feeds alone. The contribution of natural production (autotrophic and heterotrophic) to shrimp growth in experimental zero-exchange systems was determined using stable isotope techniques. Furthermore, the question of whether enhancement of shrimp growth in the presence of autotrophic populations is due to direct utilization of algae by the shrimp or the production of a growth enhancing micronutrient by the algal population is discussed.

Chapter 5 continues the examination of the role of natural production through isolation of specific amino acids from shrimp muscle tissue. Essential amino acids are

those that the shrimp are unable to produce in sufficient quantities for themselves and that must be provided by their diet. By determining the stable isotopic profiles of individual amino acids from shrimp muscle tissue it was possible to quantify the contributions of formulated feeds versus the natural pond assemblage.

A summary of the results of using stable isotope enrichments in shrimp aquaculture studies is given in Chapter 6. The dissertation concludes with suggestions for future research on this subject.

CHAPTER 2 – STABLE ISOTOPIC TECHNIQUES FOR TRACING FEED UTILIZATION

INTRODUCTION

Penaeid shrimp are popular aquacultural species, cultured throughout subtropical and tropical areas of the world, and the percentage of cultured shrimp to wild production is increasing (Van-Wormhoudt and Bellon-Humbert, 1994). Nutritional balance is one of the main challenges facing aquaculturists of this and any other species. Maximizing growth on prepared foods, however, requires detailed knowledge of the nutritional requirements of the organism cultured. In addition to nutritional value, digestibility, water stability of the formulated feed, and costs have to be taken into consideration. Akiyama et al. (1992) note, in a review of Penaeid nutrition, that commercial shrimp feeds contain varying amounts of protein, lipid, carbohydrates, vitamins, fiber and minerals. However, the requirements for these nutrients change depending on the species and size of the shrimp. For example, Lee et al. (1984) found that the protein requirements of *Penaeus vannamei* changed as the shrimp grew, with smaller shrimp requiring more animal protein. There is general information on the necessary ingredients for shrimp growth, however the optimal quantities are generally

unknown (Akiyama et al., 1992). Consequently, feeds are often formulated with more ingredients than are necessary (Goddard, 1996).

The customary technique for determining the necessary ingredients in shrimp diets is to compare growth and survival of shrimp on feed with and without a given component (D'Abramo and Castell, 1997). However, this type of study must be performed under controlled conditions in order to limit the number of variables that are present in natural systems. Yet, shrimp nutrition is supplemented with natural primary and secondary pond production under normal culture conditions, thereby making laboratory studies less applicable (Anderson et al., 1987; Nunes et al., 1997; Parker et al., 1989). It is desirable to have techniques that allow the study of nutrient flows within shrimp ponds under normal conditions. Stable isotope methodology provides a safe and effective technique to gain insight into the nutrient pathways in shrimp culture ponds.

Stable isotope ratios have been used to study food sources for a variety of organisms. Most studies have compared the natural abundance values for the diet or prey items to the isotope ratios in the target species. Since similar natural abundance in different food sources often limit the usefulness of this approach, this study sought methods for tracking nutrient flow in intensive cultures of Pacific white shrimp, *Litopenaeus vannamei*, through the addition of ^{15}N- or ^{13}C-enriched compounds to

formulated feeds. Appearance of the isotopic label was monitored in shrimp tissues as well as tank particulate matter. The goal of the study was to adapt tracer methodology for more accurate assessment of shrimp feed utilization under natural culture conditions.

MATERIALS AND METHODS

Study Location

Technique development took place during three experimental periods at the Oceanic Institute (OI), Waimanalo, Hawaii (OML 98-1, 98-5 and 99-3; Table 1.1). All experiments were carried out in outdoor mesocosm laboratories (OML) made of fiberglass that were filled with natural seawater (1200 L for OML 98-1, 1300 L for all others) from wells located at the coastline. The seawater, which is naturally filtered through a predominant basaltic substrate, is drawn from approximately 10 to 20 feet depth. After the initial filling, water was added to the tanks only to maintain the original volume. Prior to the addition of the shrimp to the tanks, water from the shrimp nursery tanks was added to initiate natural phytoplankton production, which was predominately diatoms. Tanks were stocked with juvenile *Litopenaeus vannamei* (Boone, 1931) having average weights of 1.1 to 3.5 g (Table 2.1). Water quality was monitored weekly for nitrate + nitrite and ammonia concentrations. Shrimp wet weights, feed conversion ratios (FCR; the weight of food ingested per weight gain in the shrimp), specific growth

Table 2.1: Average shrimp growth and efficiency measures for feeding experiments.

OML	Treatment	n	Initial Wt (g)	Day 1 Wt (g)	Final Wt (g)	Weight Gain (%)	Growth (g/wk)	FCR (g feed/g wet wt shrimp)	SGR (%/day)	Survival (%)
98-1	Control	4	3.36 (0.13)	3.97	8.70 (0.73)	159 (18)	0.76 (0.09)	3.36 (0.30)	1.94 (0.14)	93.6 (4.0)
	^{13}C-glycine	3	3.25 (0.25)	4.09	8.21 (0.89)	153 (13)	0.71 (0.10)	3.42 (0.45)	1.89 (0.10)	91.9 (0.9)
	^{15}N-glycine	3	3.38 (0.14)	4.09	8.62 (0.52)	155 (17)	0.75 (0.07)	3.46 (0.35)	1.91 (0.14)	91.4 (0.9)
	^{15}N-amino acid mix	3	3.32 (0.11)	4.00	9.02 (0.76)	172 (15)	0.81 (0.09)	3.20 (0.45)	2.04 (0.12)	91.9 (3.2)
98-5	Control	2	1.12 (0.01)	5.23	14.1	1170	1.08	14.4	3.02	16.7 (23.6)
	^{15}N-glycine	3	1.10 (0.03)	5.59	17.5 (0.8)	1480 (28)	1.36 (0.06)	15.4 (1.6)	3.29 (0.03)	24.4 (1.9)
	^{15}N-amino acid mix	3	1.12 (0.04)	5.31	16.5 (1.6)	1380 (165)	1.28 (0.13)	14.0 (4.5)	3.20 (0.13)	31.1 (10.7)
	^{15}N/^{13}C-algal cells	3	1.10 (0.05)	5.45	16.0 (0.1)	1390 (29)	1.24 (0.01)	13.3 (3.0)	3.22 (0.02)	21.1 (19.0)
99-3	Control	2	1.91 (0.04)	4.65	15.8 (1.0)	731 (69)	1.74 (0.13)	2.85 (0.25)	3.78 (0.15)	62.3 (1.0)
	^{15}N/^{13}C-algal cells	2	1.97 (0.02)	5.08	16.1 (0.2)	715 (18)	1.76 (0.03)	6.54 (3.45)	3.75 (0.04)	30.1 (15.5)

Numbers in ( ) represent population standard deviations.
Day 1 weights approximated from regression of weight vs. time.
n = number of tanks in trial. n = 1 tank for OML 98-5 control final weight, weight gain, growth, FCR, and SGR
n = 2 tanks for OML 98-5 ^{15}N/^{13}C algal cells final weight, weight gain, growth, FCR, and SGR

rates (SGR; percent weight gain/day), and survival for each of the tanks was routinely monitored.

Feed Preparation

All reference feeds were formulated at the Oceanic Institute (see feed formulations Appendix D). For the first two trials, OML 98-1 and 98-5, the same reference feed was used (OI Control Feed OIS971) containing 39% protein, 9% fat, and 2,500 kcal/kg of digestible energy. The reference feed in the 3rd experiment differed slightly, with 35% protein, 9.2% fat, and 2,900 kcal/kg of digestible energy (OI Control Feed LV99.HQFM.2).

Stable isotopically labeled compounds were added to the feed during mixing of the ingredients. In OML 98-1, three 15 kg batches of feed were isotopically labeled with:

(1) ^{15}N-glycine (98% ^{15}N atom enriched),
(2) ^{13}C-glycine (99% ^{13}C atom enriched), and
(3) ^{15}N-amino acid mixture (96-99% ^{15}N atom enriched).

The amino acid mixture derived from algal cell extracts (Cambridge Isotope Laboratories cat. # NLM-2161) consisted of the following amino acids by weight percent: L-Alanine (7.3%), L-Arginine (6.8%), L-Aspartic Acid (9.5%), L-Glutamic Acid (10.4%), Glycine (6.2%), L-Histidine (1.9%), L-Isoleucine (4.0%), L-Leucine (10.6%), L-Lysine (13.7%), L-Methionine (1.0%), L-Phenylalanine (4.5%), L-Proline

(6.5%), L-Serine (4.1%), L-Threonine (4.6%), L-Tyrosine (3.9%), L-Valine (5.1%). Added label represented less than 0.1% of the diet.

Experiment OML 98-5 included a repetition of the crystalline ^{15}N glycine and ^{15}N amino acid mix additions. Additionally,$^{15}N/^{13}C$ lyophilized algal cells (Cambridge Scientific CNLM-455; 96-99% ^{15}N and 98% ^{13}C atom enriched) were incorporated into feeds.

Formulated feed in OML 99-3 was isotopically labeled by incorporating $^{15}N/^{13}C$ labeled algal cells grown in culture facilities at OI. Cells of *Tetraselmis* sp., a green alga, were cultured in F/2 media (Guillard, 1975) in 20 liter culture bags under constant illumination. During the growth of these cells, 1.5 g of ^{15}N-ammonium chloride (99% ^{15}N atom enriched) and 0.625 g ^{13}C-sodium bicarbonate (99% ^{13}C atom enriched) were added to each of 4 culture bags (20 L). Air flow was reduced to retain labeled $^{13}CO_2$. The algal cells were harvested by centrifugation, freeze-dried, powdered and mixed with the ingredients of the formulated feed. ^{13}C-labeled algal cells (*Agminelum quadriplicata*) were added to further enrich the feed. (1 g of algal cells to 15 kg feed; Cambridge Isotope Laboratories, cat. # CLM-2065; 98% ^{13}C atom enriched).

Sampling

OML 98-1

Zero-time shrimp samples (3 individuals) were collected from the stock tanks on 18 March 1998 at the time the mesocosms were stocked. All treatments were assigned randomly to three tanks each. Subsequently, following acclimatization in the mesocosms, shrimp samples from each of the experimental tanks were collected on day 1 (23 March) prior to the addition of label. For recording purposes, day 1 of each of the described experiments represents the first day that labeled feeds were given to the shrimp. Shrimp were fed their respective diets from day 1 until day 22 except for ^{13}C-glycine labeled feed, which was continued to the end of the experiment. The shrimp were initially fed at a rate of 6% of their biomass per day, with this rate declining over the course of the experiment to a final value of 2.5%. Declines in shrimp numbers due to sampling or observed mortalities were incorporated into calculation of feed amounts. Shrimp were collected on days 3 and 5, then at weekly intervals until the end of the experiment. Samples of tank suspended particulate matter (SPOM) were taken at the same time as the shrimp by filtering 10 to 20 ml of water through pre-combusted 25 mm GF/F filters. The ranges of material appearing on the filters, as well as an example of its changes over time, are given in Appendix A.

OML 98-5

As above, each treatment was randomly assigned to three tanks. Three shrimp were sampled from each tank on 7 October 1998 for zero-time analysis. Labeled feeds were given after this sampling and continued until 9 December 1998. Shrimp were fed 3 times daily at a declining rate of 8% to 2.5% of their body weight during the course of the experiment. Shrimp were sampled twice a week (3/tank) and frozen to preserve them for stable isotopic analysis. Samples of tank suspended particulate organic matter (SPOM) were collected as in OML 98-1.

OML 99-3

Each treatment was again randomly assigned to three tanks. In OML 99-3, zero-time samples were collected on 2 August 1999 and labeled feeds were started after initial sample collection. Shrimp were fed labeled feeds until day 36, when they again received control diets. Shrimp (3/tank) were sampled biweekly until 14 September 1999. As with OML 98-5, shrimp were again fed 3 times daily at a declining rate of 8% to 2.5% of their body weight during the course of the experiment. These samples were frozen for subsequent stable isotopic analysis. Two shrimp from replicate tanks were used for isotopic determination. Samples of tank suspended particulate organic matter were also taken biweekly. From the start of the experiment until day 15, 5 to 40 ml of water were filtered through pre-combusted GF/F filters. From day 18 until the end of

the experiment, 100 ml of water was centrifuged before being filtered through the GF/F filter to limit clogging.

Sample Preparation

OML 98-1

Collected shrimp samples were boiled for 5 minutes to denature enzymes and then dried at 65-80°C to constant weight. Filters for SPOM analysis were acid fumed by placing them in an vacuum sealed container with a small beaker containing 20 ml of 50% HCl overnight for removal of carbonates and then dried.

OML 98-5 and OML 99-3

Shrimp samples from the second (OML 98-5) and third (OML 99-3) trials were separated into carapace and muscle tissues prior to mass spectrometry. Muscle samples were rinsed in deionized water (DI) and dried in an oven (80°C) for 48 hours. Carapace samples were rinsed in DI and acid fumed as described above to remove carbonates. These samples were then dried and subsamples taken for mass spectrometer analysis. The possibility of bacterial assemblages being attached to the carapace in sufficient quantities as to affect the isotopic values was tested by determining isotopic ratios for carapace samples that were scrubbed and unscrubbed prior to acidification. A paired t-test indicated no difference between the treatments and thus carapace values described

reported are from unscrubbed samples (see Appendix B). Filters were prepared as in OML 98-1.

Mass Spectrometer Analysis

Dried muscle samples and feed samples were ground into a fine homogenous powder before mass spectrometer analysis. Carapace material was subsampled without homogenization. SPOM isotopic ratios were determined by analyzing whole or half filters depending on the sample amount. Isotopic ratios were obtained using either a Europa 20/20 continuous flow isotope ratio mass spectrometer (all OML 98-1 samples as well as muscle, carapace, and feed samples from OML 98-5) or a Finnigan Delta Plus mass spectrometer (all remaining samples). Stable isotope results are presented as atom percent excess or in standard delta notation where:

$$\delta(‰) = (R_{sample} - R_{std}) / R_{std} \times 1000$$

and R is the ratio of heavier to light isotope of sample and standard respectively. Standards were a peptone for both carbon and nitrogen, referenced to Vienna Pee Dee Belemnite for carbon and air for nitrogen.

Calculations

Percent Label Reflected in Muscle

The amount of ^{15}N in shrimp muscle tissue was compared to the amount added via labeled feed to determine how much of the feed's isotopic label was reflected in the

shrimp. The difference in ^{15}N abundance (atom percent excess) between control and experimental shrimp was calculated. Using the measured wet weight-dry weight ratio and the percent of nitrogen in the sample, it was possible to multiply the atom percent excess ^{15}N by the amount of nitrogen and obtain a mass value for ^{15}N in the shrimp muscle. In the case of the feeds, the amount of ^{15}N was calculated similarly. Briefly, atom percent excess was calculated by subtracting the abundance of ^{15}N in reference feed from that in labeled feed. The amount of feed given to each shrimp daily was calculated by dividing the amount of feed added to a tank by the theoretical number of shrimp remaining. The amount of nitrogen available daily in the feed was multiplied by the atom percent excess ^{15}N in the feed to obtain the weight of feed ^{15}N. The daily values were added to account for the repeated addition of label to the tanks. The mass values for ^{15}N in the shrimp muscle and the feed were compared to approximate the amount of label assimilated by the shrimp (See Appendix C for a sample calculation).

Statistics

Differences in muscle and carapace $\delta^{13}C$ and $\delta^{15}N$ values for individual shrimp were compared using paired Student's t tests where $p \leq 0.05$ was considered statistically significant (i.e. at a 5% significance level). Statistical analysis of the response by the different compartments (muscle, carapace, and SPOM) to the various treatments was

done by comparing slopes from the regression of $\delta^{13}C$ or $\delta^{15}N$ on day. The slopes of two lines were compared using the following equation:

$$t = (b_1 - b_2)/SQRT(SE_{b1}^2 + SE_{b2}^2)$$

where b_1 and b_2 are the slopes of the two lines being compared and SE is the standard error of the regression line (Fowler et al., 1998). An overall significance level of 0.05 was chosen, but divided by the number of t-tests performed (Bonferroni multiple comparisons procedure) to limit the possibility of Type I error (Mendenhall and Sincich, 1996). The comparisons of slope were only performed between the control and each of the treatments for a given experiment to satisfy the assumption of independence. Changes in $\delta^{13}C$ or $\delta^{15}N$ over time among the label addition treatments were not compared by this means because the level of enrichment differed among treatments, and thus their slopes are not truly comparable. These treatments were compared using percent label values. One-way ANOVA was used to compare growth, survival, feed conversion ratio (FCR), specific growth rate (SGR) and percent label values. For ANOVA, individual shrimp values were averaged for a tank mean value before using tank values as a repeated measure. A Tukey test for multiple comparisons was used in association with the one-way ANOVA to examine specific means for statistical differences. FCR values were log transformed prior to one-way ANOVA due to

increasing variance with increasing values. Values throughout were reported as mean ± population standard deviation.

RESULTS

Growth and Efficiency Measures

OML 98-1

Table 2.1 lists the growth and efficiency measures for the shrimp receiving control feed and feeds with isotopic labels for each of the first 3 trials. The average growth of the shrimp in OML 98-1 was 0.7 to 0.8 g/week and survival was 91.4% to 93.6%. Growth rates overall were lower than known maxima, presumably due to cooler ambient temperatures (morning = 24.1 ± 1.2°C [mean ± population standard deviation] and afternoon = 26.2 ± 1.3°C) during the experiment. The feed conversion ratio (FCR) ranged from 3.2 to 3.5 g feed/g wet weight of shrimp, and specific growth rate (SGR) was approximately 2.0 %/day. There was no significant difference between OML 98-1 treatments (one-way ANOVA; $p > 0.05$) for any of these performance measurements.

OML 98-5

Mean growth for shrimp in OML 98-5 ranged from 1.1 to 1.4 g/week, but survival was lower (16.7 to 31.1%; Table 2.1). Specific growth rates were 3.0 to 3.3%/day. The average feed conversion ratios (FCR) were high (13.3 to 15.4 g feed/g wet wt of shrimp). There was no statistical difference between OML 98-5 treatments for

any of the efficiency measures. In general, weekly growth, FCR, and SGR were higher in OML 98-5 tanks than in OML 98-1, while survival was significantly lower (one-way ANOVA combined with Tukey procedure; $p < 0.001$).

OML 99-3

In OML 99-3, shrimp growth averaged 1.7 to 1.9 g/week (Table 2.1). The average FCR values ranged from 2.9 to 6.5 g feed/g wet weight of shrimp. SGR values were 3.8%/day. Survival was quite variable and low with average values ranging between 30.1% to 62.3%. There were no statistical differences in the efficiency measures between treatments ($p > 0.05$). When compared to the previous 2 experiments, OML 99-3 weekly growth rates and SGR were statistically higher. FCR was similar to OML 98-1 and less than OML 98-5. Survival rates were slightly higher than OML 98-5, but significantly lower than those in OML 98-1 (one-way ANOVA combined with Tukey procedure; $p<0.05$).

Nutrients

Figures 2.1 to 2.3 show the trends in nitrate (NO_3^-) plus nitrite (NO_2^-), and total ammonia nitrogen (TAN; ammonium NH_4^+ and ammonia NH_3) for each of the tanks in each trial. In each trial, TAN concentrations decreased over time while $NO_3^- + NO_2^-$ concentrations increased indicating nitrification within the tanks. No significant

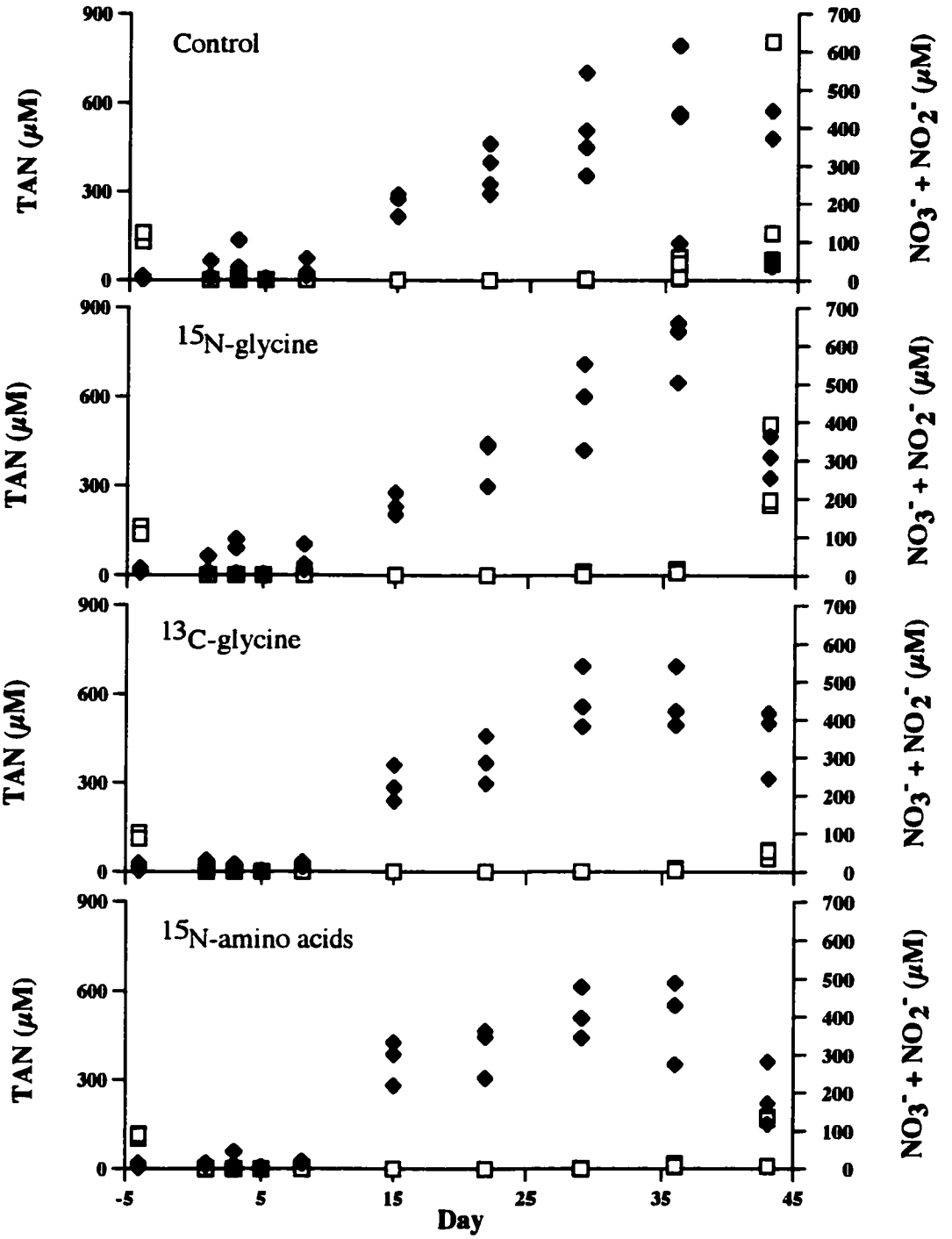

Figure 2.1: Nutrient profiles for OML 98-1. □ $NO_3^- + NO_2^-$; ◆ TAN.

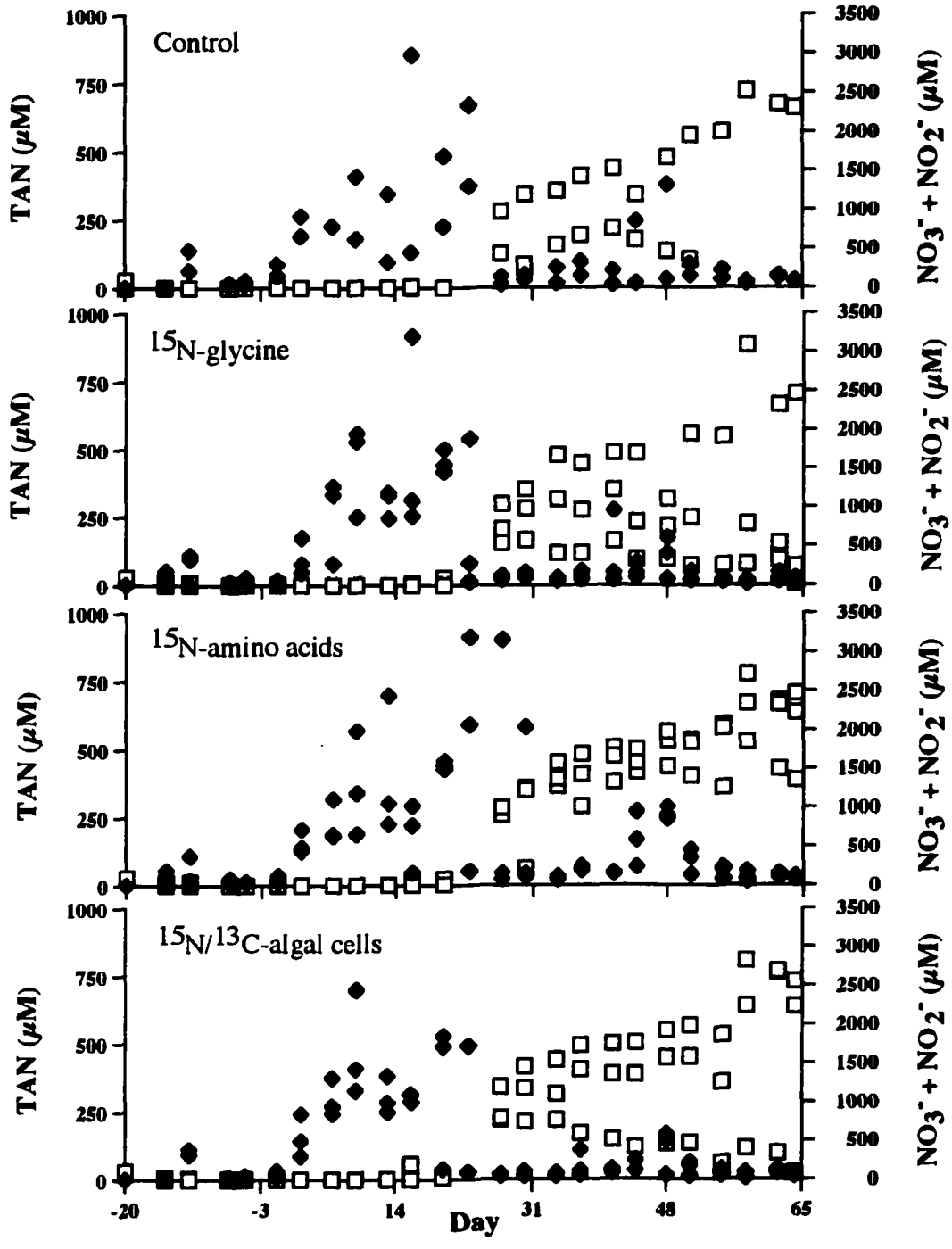

Figure 2.2: Nutrient profiles for OML 98-5. □ $NO_3^- + NO_2^-$; ◆ TAN.

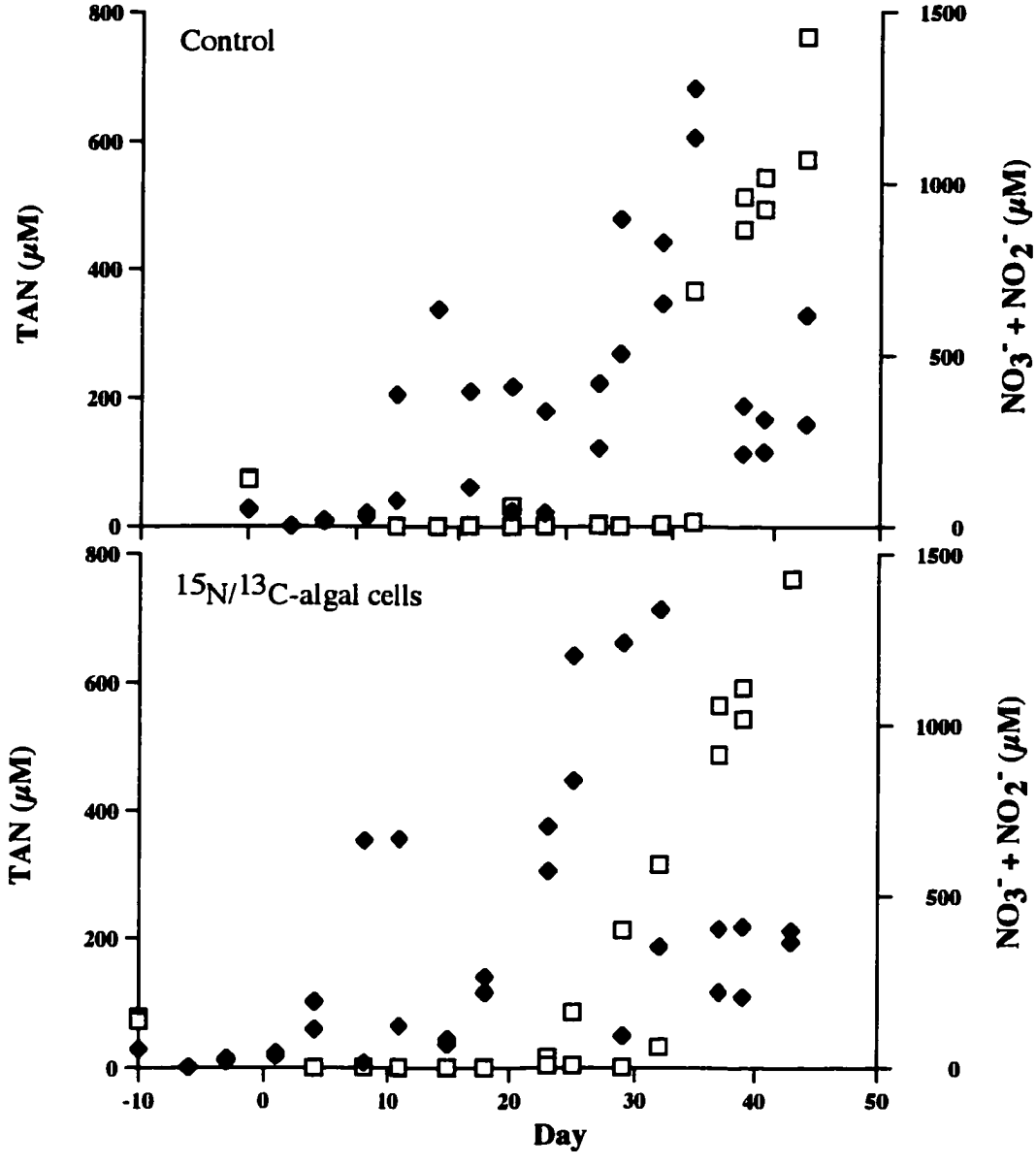

Figure 2.3: Nutrient profiles for OML 99-3. □ $NO_3^- + NO_2^-$; ◆ TAN.

differences were noted for either of the nutrient measures between the control tanks of each trial and the labeled feed tanks.

General Isotopic Trends from Control Tanks

OML 98-1

The mean $\delta^{13}C$ and $\delta^{15}N$ values for shrimp and SPOM in the control tanks are given in Figure 2.4. The $\delta^{15}N$ values for the shrimp muscle increased slightly over the course of the experiments from averages of 9.3‰ on day 1 to 11.0‰ on day 43. While the carapace samples were significantly lower in $\delta^{15}N$ than the muscle (paired t test; $p \leq 0.001$), all components became slightly higher in ^{15}N values over the course of the experiment. The $\delta^{15}N$ value for suspended particulate matter showed large variability, particularly early in the experiments, which may have been due to the small amounts of material on the filters and consequent lowered precision in the mass spectrometry. Overall, the particulate nitrogen values generally increased during the course of the experiment (from 8.4‰ to 12.3‰).

$\delta^{13}C$ values for the control tanks showed minor isotopic changes for both the muscle and carapace samples. Muscle samples increased from averages of -19.4‰ to −18.9‰ from day 1 to day 43 respectively. The carapace samples averaged −18.8‰ and −18.7‰ on days 1 and 43. Carapace samples were slightly enriched in ^{13}C relative to

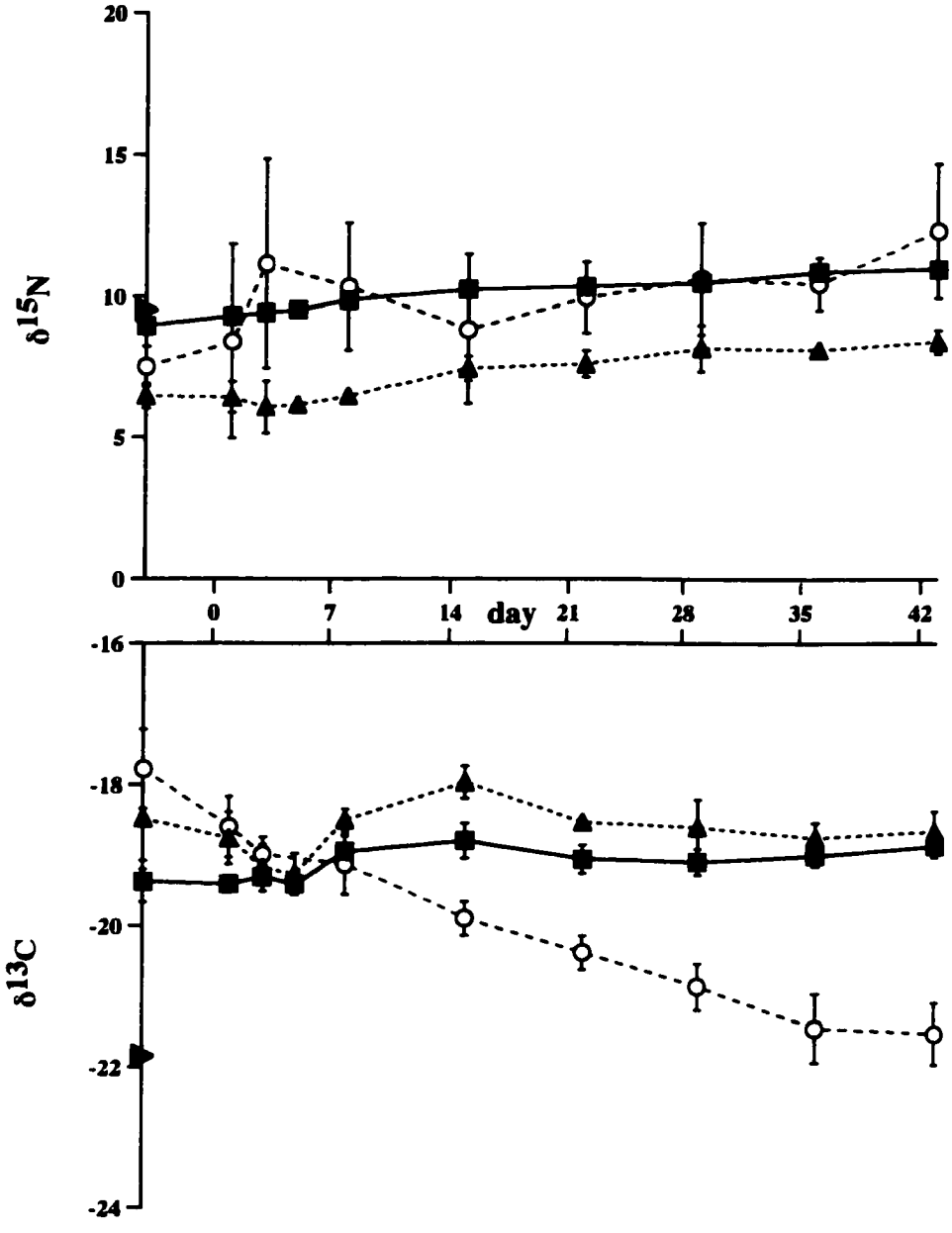

Figure 2.4: Natural abundance $\delta^{15}N$ and $\delta^{13}C$ values for control tanks in OML 98-1. Samples of shrimp muscle and carapace and SPOM were taken from 4 tanks for each indicated day. Error bars represent population standard deviations. ■ shrimp muscle; ▲ shrimp carapace; O SPOM; ▸ feed.

muscle (paired t test; $p \leq 0.001$), but the differences between the two tissue types were less than 1‰. Suspended particulate samples, on the other hand, decreased in $\delta^{13}C$ over the course of the experiment (average values of -18.6‰ to –21.5‰).

OML 98-5

OML 98-5 control tank $\delta^{15}N$ and $\delta^{13}C$ values are shown in Figure 2.5. Shrimp carapace and muscle $\delta^{15}N$ values did not change appreciably over the course of the experiment (10.8 to 11.2‰ from day 1 to day 62 for muscle and 7.7 to 8.5‰ for carapace). As with OML 98-1, carapace $\delta^{15}N$ values were significantly lower than muscle values (paired t-test; $p \leq 0.001$) by approximately 3‰. SPOM samples increased in $\delta^{15}N$ over the course of the experiment (mean = 8.1‰ on day –1 to 13.9‰ on day 62).

The $\delta^{13}C$ values for carapace and muscle also changed only slightly over the experiment (-18.6 to –18.9‰ for muscle and –17.8 to –19.7‰ for carapace), but unlike OML 98-1 they were not significantly different from each other (paired t-test; $p = 0.88$). SPOM samples decreased in $\delta^{13}C$ over time with the exception of day 42 (-24.3‰ on day 62 compared to a feed value of –21.9‰).

OML 99-3

Control tank $\delta^{15}N$ and $\delta^{13}C$ values for OML 99-3 are shown in Figure 2.6. As

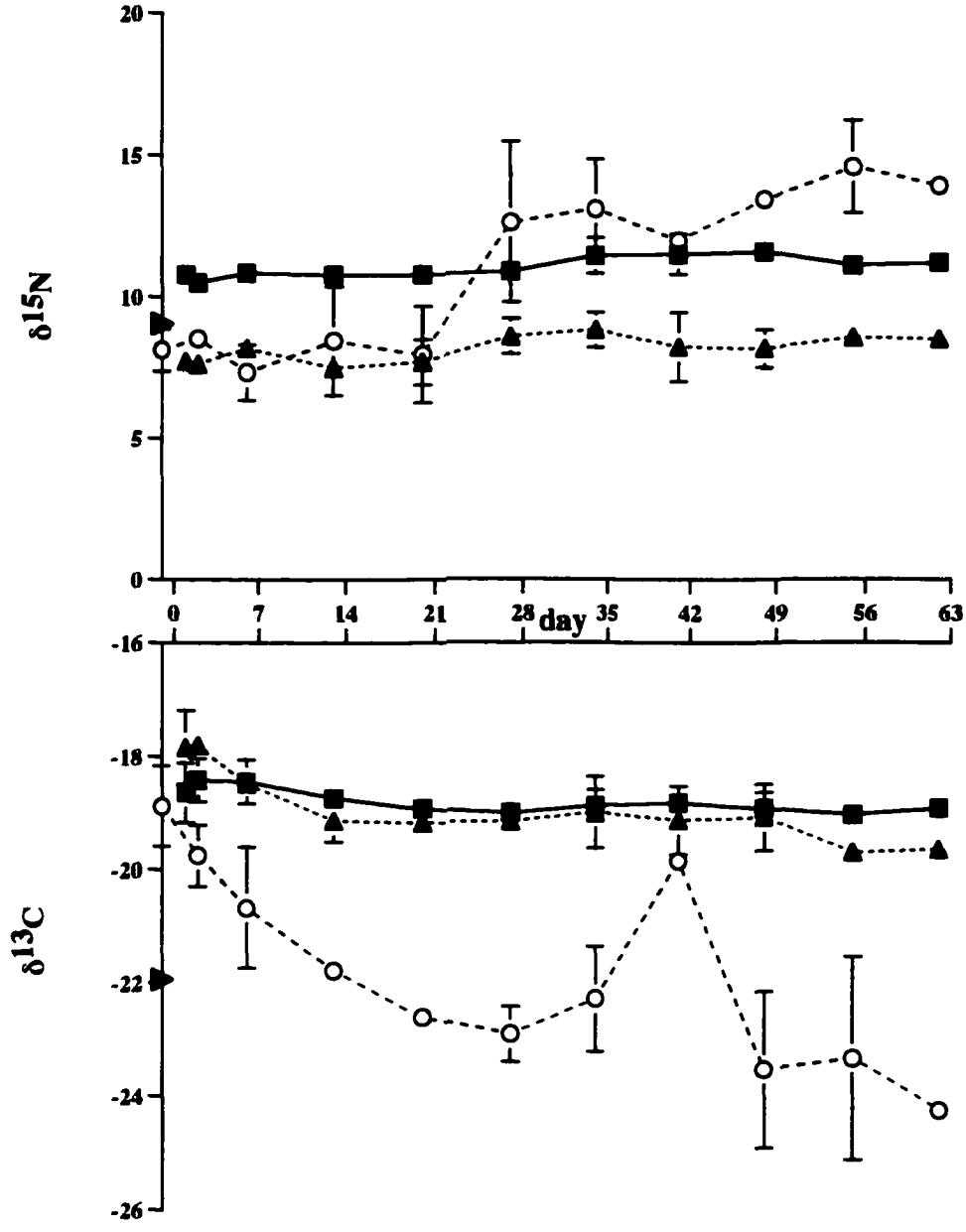

Figure 2.5: Natural abundance $\delta^{15}N$ and $\delta^{13}C$ values for control tanks in OML 98-5. Samples of shrimp muscle and carapace and SPOM were taken from 2 tanks for each indicated day (except n = 1 for muscle and carapace, day 55 and SPOM, day 41). Error bars represent population standard deviations. ■ shrimp muscle; ▲ shrimp carapace; O SPOM; ▸ feed.

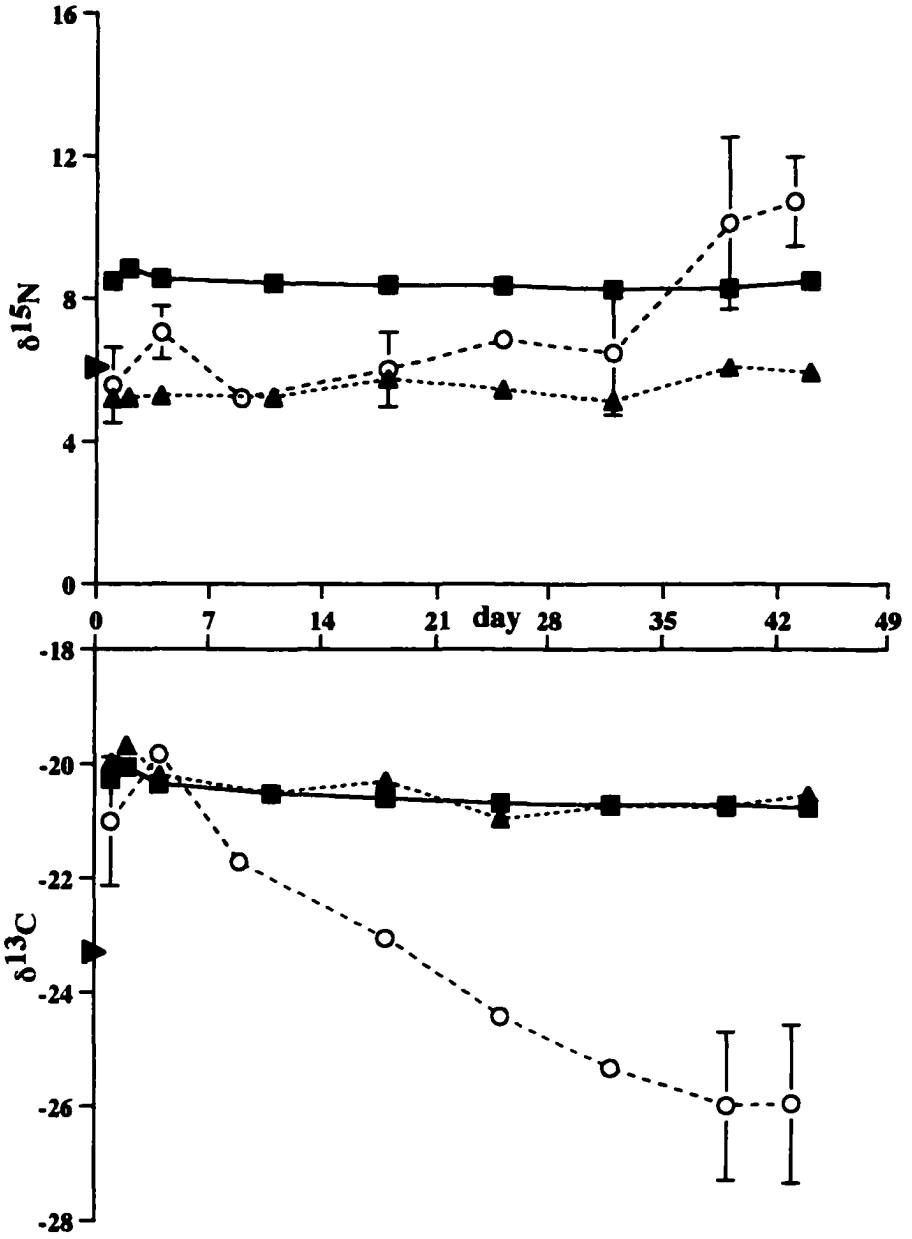

Figure 2.6: Natural abundance $\delta^{15}N$ and $\delta^{13}C$ values for control tanks in OML 99-3. Samples of shrimp muscle and carapace and SPOM were taken from 2 tanks for each indicated day. Error bars represent population standard deviations. ■ shrimp muscle; ▲ shrimp carapace; O SPOM; ▸ feed.

with the previous trial, shrimp carapace and muscle $\delta^{15}N$ values did not change appreciably over the course of the experiment (average values of 8.5 to 8.5‰ from day 1 to day 44 for muscle and 5.2 to 6.0‰ for carapace). Again, carapace values were significantly lower than muscle values (paired t-test; $p \leq 0.001$) by approximately 3‰. SPOM samples increased in $\delta^{15}N$ over the course of the experiment (average values of 5.6‰ to 10.7‰). The average $\delta^{13}C$ values for carapace and muscle also changed only slightly over the experiment (-20.3 to -20.8‰ for muscle and -20.0 to -20.5‰ for carapace). Muscle and carapace $\delta^{13}C$ values were not statistically different (paired t test; $p = 0.16$). SPOM samples decreased in $\delta^{13}C$ over time from an average of –21.0‰ to –26.0‰ (feed value = -23.3‰).

Feeding Experiments: Crystalline Amino Acids

^{13}C-Glycine Labeled Feed

In the ^{13}C-glycine experiment performed during OML 98-1 (Figure 2.7), the nitrogen isotope values (no label added) tracked samples from the reference tanks very closely (Figure 2.4). The change in $\delta^{15}N$ from day 1 to day 22 for muscle, carapace, and SPOM was not significantly different from the OML 98-1 controls. For carbon, however, the $\delta^{13}C$ of muscle, carapace, and particulate samples increased in apparent response to the added labeled feeds, which had $\delta^{13}C = -13.6‰$. While the starting $\delta^{13}C$

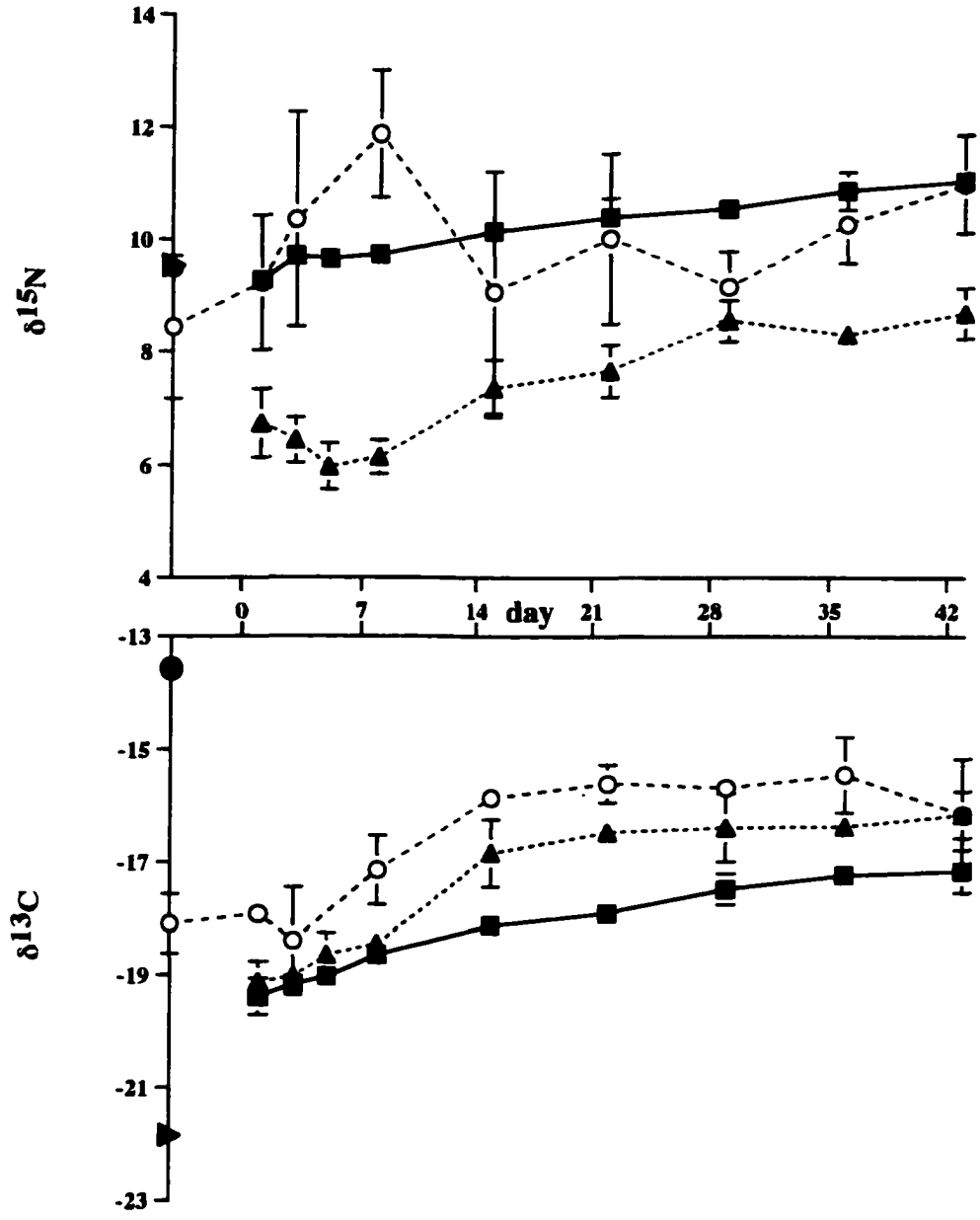

Figure 2.7: $\delta^{15}N$ and $\delta^{13}C$ values for tanks receiving ^{13}C-glycine amended feed in OML 98-1. Shrimp were fed labeled feed from day 1 until the end of the experiment. Samples of shrimp muscle and carapace and SPOM were taken from 3 tanks for each indicated day. Error bars represent population standard deviations. ■ shrimp muscle; ▲ shrimp carapace; O SPOM; ▸ initial feed; • labeled feed.

values were similar to the reference values, the increase in $\delta^{13}C$ over the 22 days of label addition for muscle, carapace, and suspended particulate matter contrasted significantly with the control values (slope analysis; overall $p < 0.05$, individual comparisons $p < 0.003$). In this trial, as in each of the label addition experiments, the isotope ratios for the carapace responded more quickly to the new feed signal than did the muscle ratios. Isotopic values for SPOM in all labeled feed experiments showed close agreement with the isotopic label of the feed, either due to rapid recycling or inclusion of the feed pellets in SPOM.

^{15}N-Glycine Labeled Feed

OML 98-1

The shrimp in tanks that received feed augmented with ^{15}N-glycine had muscle, carapace, and particulate $\delta^{13}C$ signals (Figure 2.8) which were not significantly different from those of the reference tanks from the same trial (Figure 2.4). However, the $\delta^{15}N$ values for all three sample types showed clear increases over the period of addition. Average muscle and carapace values increased toward the labeled feed value until the end of label addition (day 22 muscle = 12.8‰, carapace = 14.8‰, and feed = 29.6‰), while SPOM values nearly matched that of the feed (28.1‰). Slope comparisons indicated that the increase in muscle, carapace, and SPOM $\delta^{15}N$ over the period of label

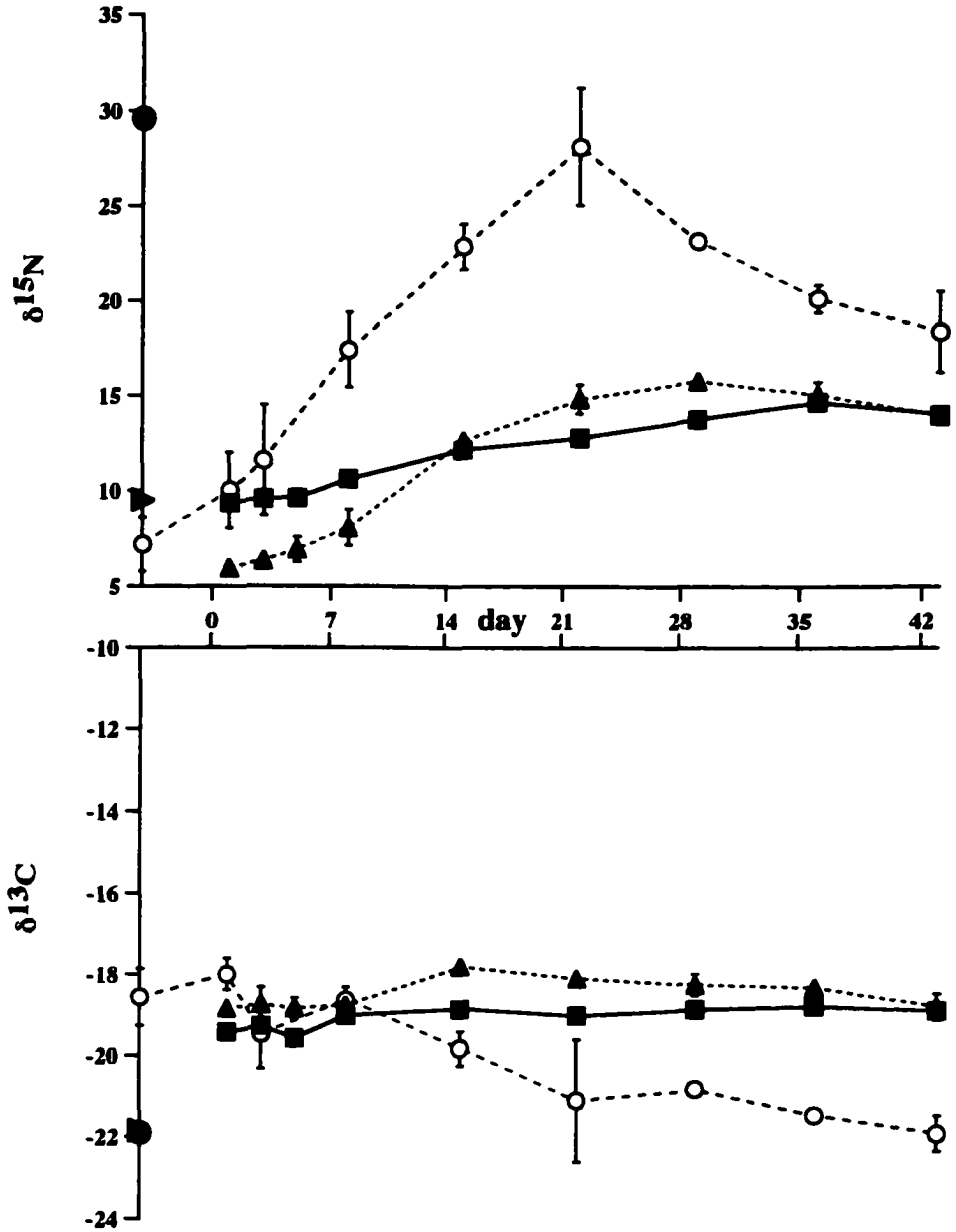

Figure 2.8: $\delta^{15}N$ and $\delta^{13}C$ values for tanks receiving ^{15}N-glycine amended feed in OML 98-1. Shrimp were fed labeled feed from day 1 to day 22. Samples of shrimp muscle and carapace and SPOM were taken from 3 tanks for each indicated day. Error bars represent population standard deviations. ■ shrimp muscle; ▲ shrimp carapace; O SPOM; ▸ initial feed; • labeled feed.

addition was significantly higher than that of the OML 98-1 controls (slope analysis; overall $p < 0.05$, individual comparisons $p < 0.003$).

OML 98-5

The $\delta^{15}N$ values for shrimp muscle and carapace tissue in the ^{15}N-glycine treatment from OML 98-5 trended similarly to OML 98-1 and increased toward the value of the labeled feed (day 62 mean muscle = 19.8‰; carapace = 20.7‰; feed = 44.9‰; Figure 2.9). The maximal value of SPOM $\delta^{15}N$ (mean $\delta^{15}N$ = 51.8‰ on day 55) was greater than that of the labeled feed, indicating preferential incorporation of the glycine nitrogen. This increase in $\delta^{15}N$ for muscle, carapace, and SPOM over the experimental period was significantly greater than that found for OML 98-5 control samples (slope analysis; overall $p < 0.05$, individual comparisons $p < 0.003$). The $\delta^{13}C$ values for carapace, muscle, and SPOM samples were similar to those for the control tanks.

Mixed ^{15}N-Amino Acid Feed

OML 98-1

For the tanks receiving feed with mixed ^{15}N-amino acids (Figure 2.10), the $\delta^{13}C$ values for all components sampled remained similar to the controls. $\delta^{15}N$ for muscle and carapace increased gradually over the period of label addition (mean $\delta^{15}N$ = 9.5‰ to 16.0‰ and 5.9‰ to 20.6‰ respectively from day 1 to day 22) toward the feed value of

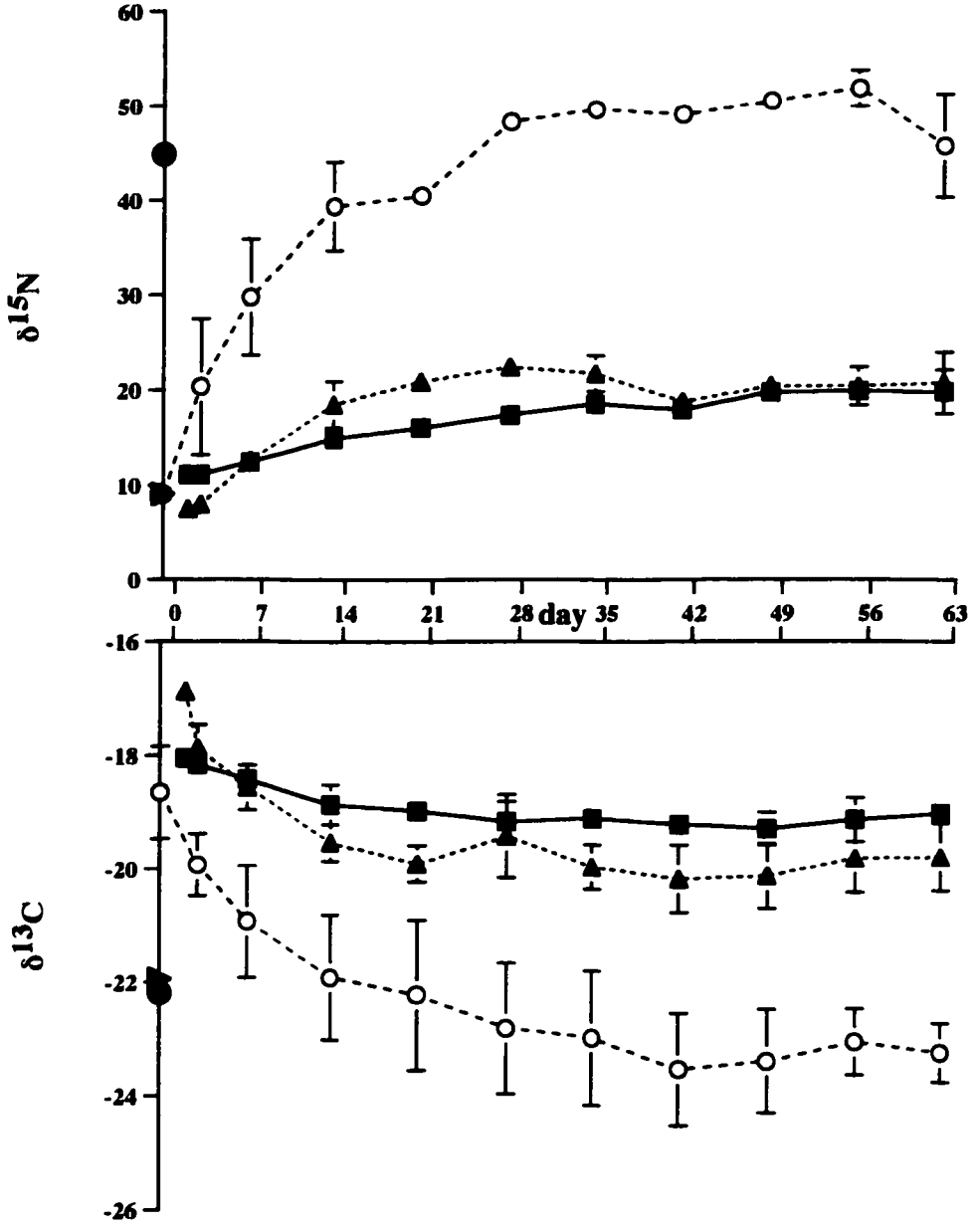

Figure 2.9: $\delta^{15}N$ and $\delta^{13}C$ values for tanks receiving ^{15}N-glycine amended feed in OML 98-5. Shrimp were fed labeled feed from day 1 until the end of the experiment. Samples of shrimp muscle and carapace and SPOM were taken from 3 tanks for each indicated day. Error bars represent population standard deviations. ■ shrimp muscle; ▲ shrimp carapace; O SPOM; ▸ initial feed; • labeled feed.

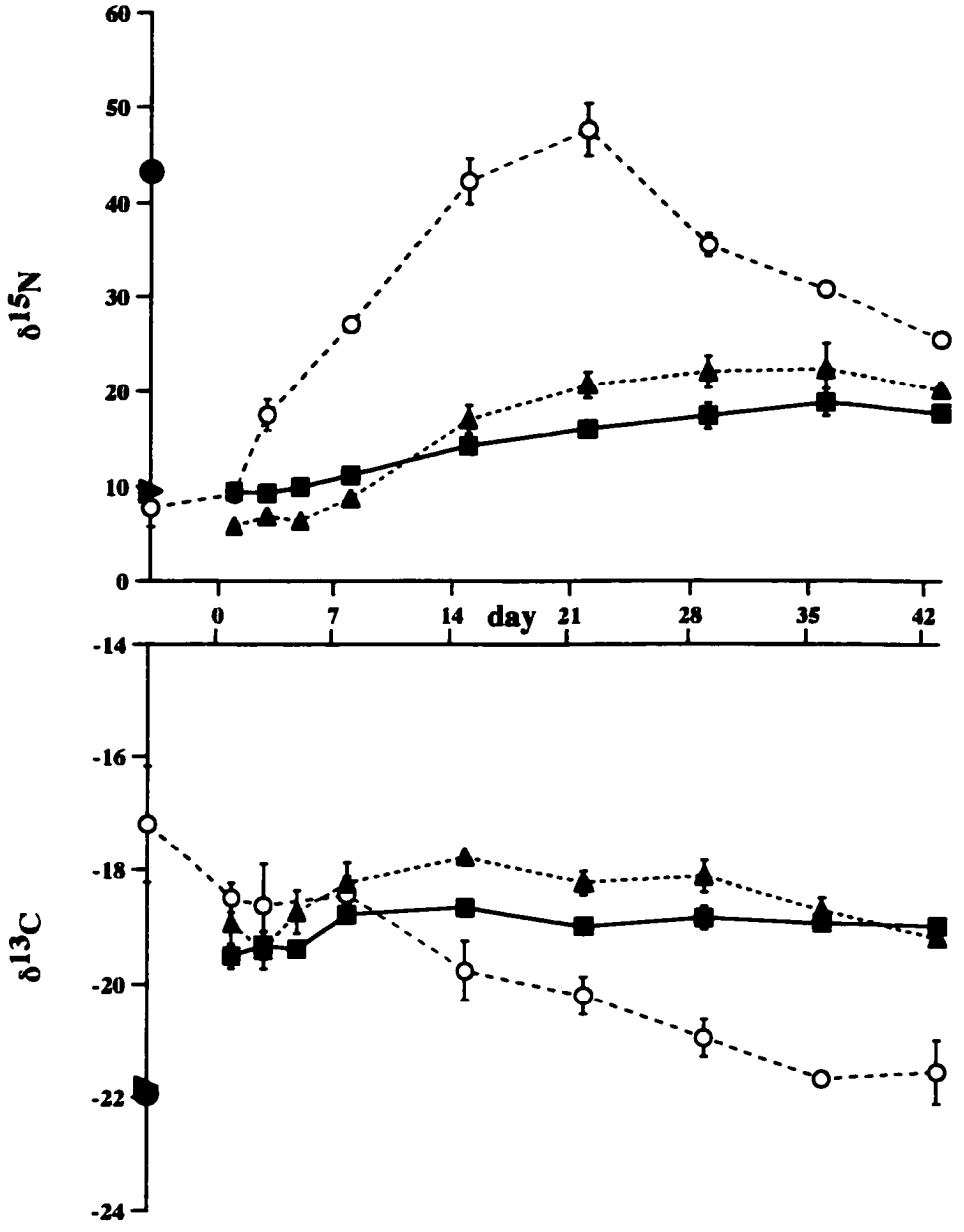

Figure 2.10: $\delta^{15}N$ and $\delta^{13}C$ values for tanks receiving ^{15}N-amino acid mix amended feed in OML 98-1. Shrimp were fed labeled feed from day 1 to day 22. Samples of shrimp muscle and carapace and SPOM were taken from 3 tanks for each indicated day. Error bars represent population standard deviations. ■ shrimp muscle; ▲ shrimp carapace; O SPOM; ▸ initial feed; • labeled feed.

43.3‰. As in the previous label addition experiments, the increase in SPOM $\delta^{15}N$ was much greater than muscle and carapace increases, with values matching the feed by the end of the addition (47.6‰). The increase in $\delta^{15}N$ for muscle, carapace, and SPOM over the period of label addition was significantly greater than that found for OML 98-1 controls (slope analysis; overall $p < 0.05$, individual comparisons $p < 0.003$).

OML 98-5

Shrimp samples for the ^{15}N-amino acid mix treatment in OML 98-5 showed a much larger increase in $\delta^{15}N$ over the course of the experiment than did samples from the same treatment in OML 98-1, due to the larger isotopic enrichment of the feed (81.2‰). Carapace samples increased from mean $\delta^{15}N$ values of 7.5‰ to 33.4‰ by day 62, while muscle increased from 10.9‰ to 29.7‰ (Figure 2.11). SPOM values increased to a mean of 84.1‰ by the end of the label addition. Carapace, muscle and SPOM $\delta^{15}N$ increases over time were significantly different from OML 98-5 controls (slope analysis; overall $p < 0.05$, individual comparisons $p < 0.003$). As expected, all $\delta^{13}C$ values were similar to those of the control tanks.

Feeding Experiments: $^{15}N/^{13}C$-Algal Cell Labeled Feed

OML 98-5

The incorporation of whole lyophilized $^{15}N/^{13}C$-algal cells to the feeds resulted

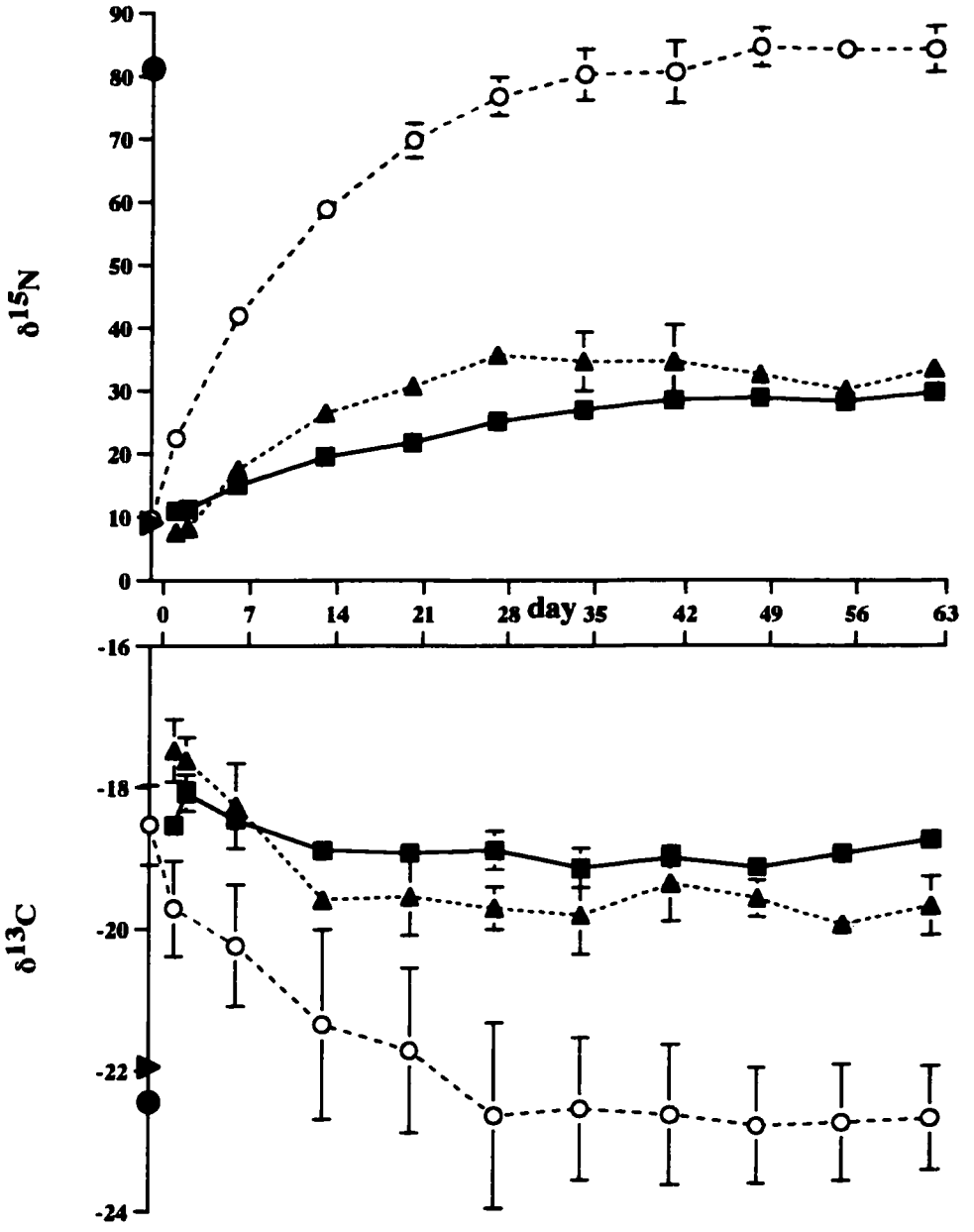

Figure 2.11: $\delta^{15}N$ and $\delta^{13}C$ values for tanks receiving ^{15}N-amino acid mix amended feed in OML 98-5. Shrimp were fed labeled feed from day 1 until the end of the experiment. Samples of shrimp muscle and carapace and SPOM were taken from 3 tanks for each indicated day. Error bars represent population standard deviations. ■ shrimp muscle; ▲ shrimp carapace; O SPOM; ▸ initial feed; • labeled feed.

in carapace and muscle $\delta^{15}N$ and $\delta^{13}C$ values that indicated much more efficient incorporation of the feed label than the treatments incorporating crystalline compounds. Muscle $\delta^{15}N$ averaged 11.1‰ to 31.6‰, while the carapace averaged 7.7‰ to 35.3‰, and the feed was 41.7‰ (Figure 2.12). The maximum carapace and muscle values of $\delta^{13}C$ (-13.9‰ and –13.7‰, respectively) exceeded that for the feed (-15.4‰). Carapace was more enriched than muscle for both $\delta^{15}N$ and $\delta^{13}C$ (paired t test; $p \leq 0.001$ for both $\delta^{15}N$ and $\delta^{13}C$). SPOM also showed a dramatic increase in $\delta^{15}N$ and $\delta^{13}C$, with values near those of the feed (maximum average values of 50.3‰ for $\delta^{15}N$ and –16.6‰ for $\delta^{13}C$). The increase in $\delta^{15}N$ and $\delta^{13}C$ for all sampled components over the experiment was significantly different from OML 98-5 control tanks (slope analysis; overall $p < 0.05$, individual comparisons $p < 0.003$).

OML 99-3

When algal cells were added to the formulated feed in OML 99-3, the final nitrogen isotope ratio of the feed was much higher than in OML 98-5 ($\delta^{15}N = 332.4$‰ and $\delta^{13}C = -15.8$‰). As in OML 98-5, shrimp muscle and carapace values, as well as SPOM, showed rapid assimilation of the $^{15}N/^{13}C$ labeled algal cells (Figure 2.13). Maximum average $\delta^{15}N$ values were 193.9‰ for muscle, 232.6‰ for carapace and 253.1‰ for SPOM. The $\delta^{13}C$ for the shrimp muscle and carapace and tank SPOM again

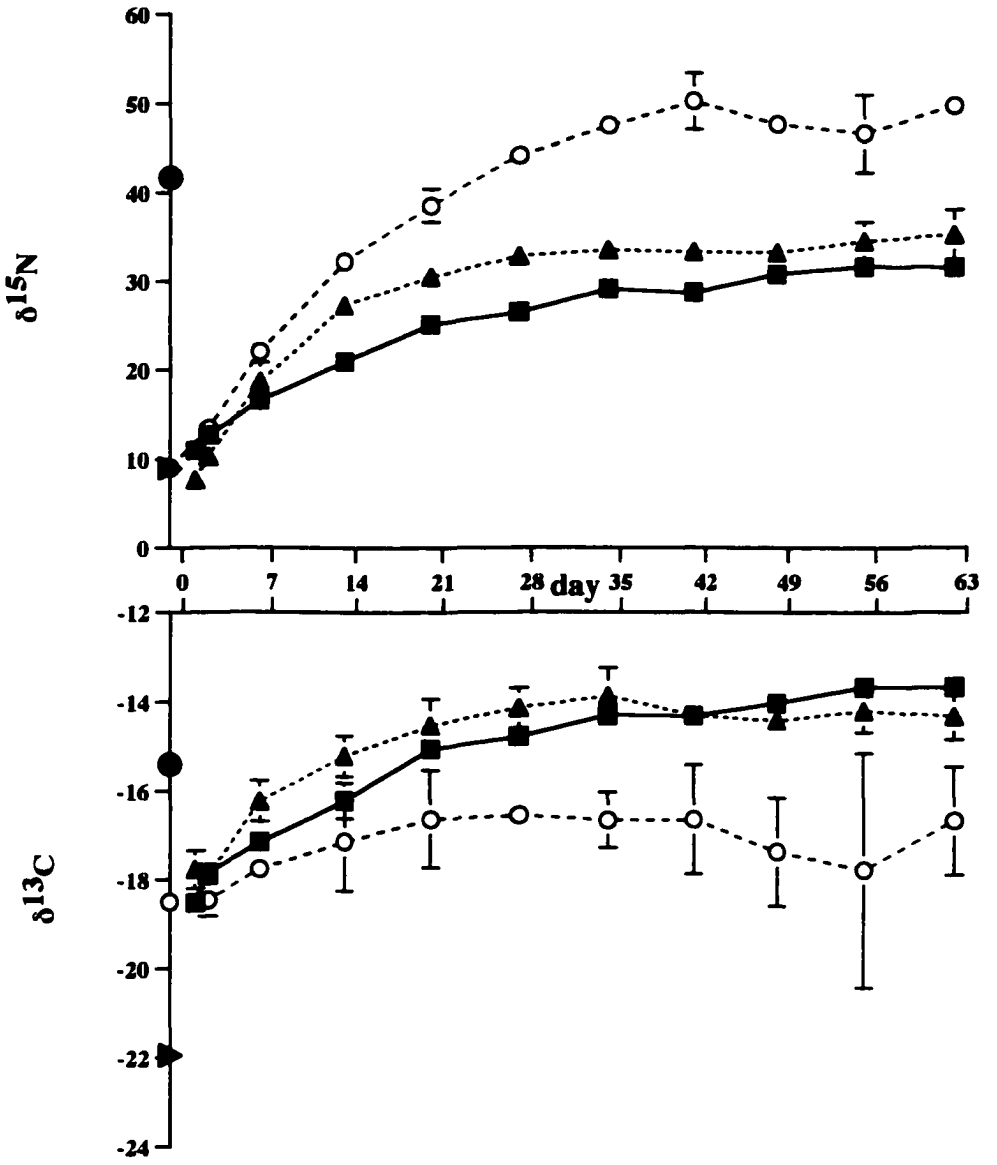

Figure 2.12: $\delta^{15}N$ and $\delta^{13}C$ values for tanks receiving $^{15}N/^{13}C$-algal cell amended feed in OML 98-5. Shrimp fed labeled feed from day 1 until end of experiment. Samples of shrimp muscle and carapace and SPOM were taken from 3 tanks for each indicated day (except n = 2 for muscle and carapace, days 41 and 48 and SPOM day 62 and n = 1 for day 27 $\delta^{13}C$). Error bars represent population standard deviations. ■ shrimp muscle; ▲ shrimp carapace; O SPOM; ▸ initial feed; • labeled feed.

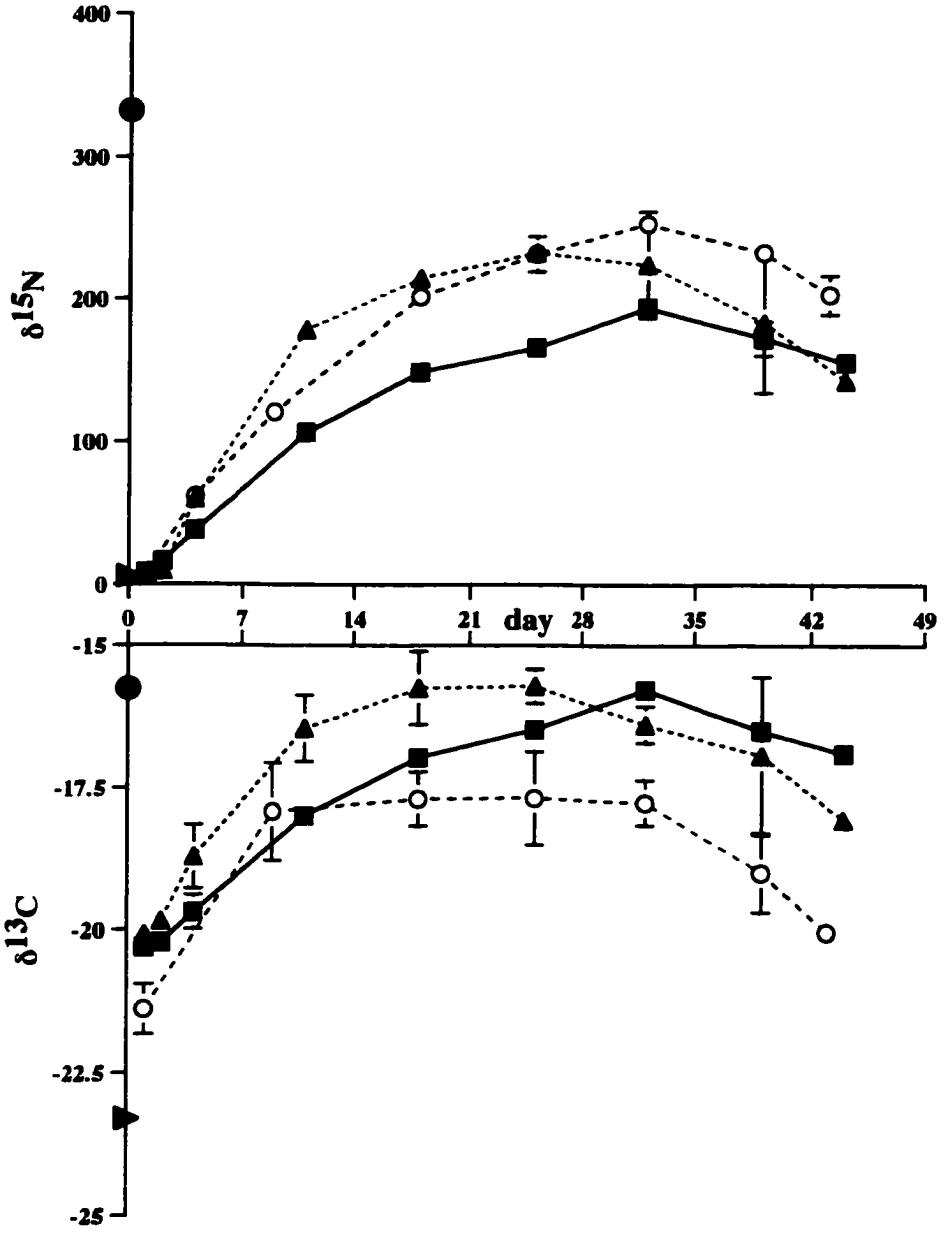

Figure 2.13: $\delta^{15}N$ and $\delta^{13}C$ values for tanks receiving $^{15}N/^{13}C$-algal cell amended feed in OML 99-3. Shrimp were fed labeled feed from day 1 to day 36. Samples of shrimp muscle and carapace and SPOM were taken from 2 tanks for each indicated day. Error bars represent population standard deviations. ■ shrimp muscle; ▲ shrimp carapace; O SPOM; ▸ initial feed; • labeled feed.

closely matched the feed value (-15.8‰, -15.7‰, and –17.7‰ respective averages). These increases were significantly different than those of control tanks (slope analysis; overall $p < 0.05$, individual comparisons $p < 0.008$). When the shrimp received unlabeled feeds on day 36, each sampled compartment decreased in both $\delta^{15}N$ and $\delta^{13}C$ toward the new feed values of 6.1‰ and –23.3‰, respectively.

Feeding Experiments: Percent Label Reflected in Muscle

Crystalline Amino Acids

The percentage of label reflected in the muscle tissue was low in the experiments using crystalline amino acids as the isotopic feed label (Table 2.2). Due to the different lengths of time that labeled feed was offered to the shrimp, statistical analysis was performed for values calculated after approximately 3 weeks of addition (the maximal time that labeled feed was given to shrimp in OML 98-1). After 22 days of label addition in OML 98-1, muscle tissue in ^{13}C-glycine and ^{15}N-glycine addition trials reflected an average of 4.1 ± 0.3% and 6.4 ± 1.2% of their respective labels. Statistical analysis indicated that ^{15}N-glycine percent label was not significantly different from the ^{13}C-glycine percent label. The ^{15}N-amino acid mix experiment showed values that were not significantly different from the ^{15}N-glycine experiment, with an average percent label of 9.4 ± 2.0%. In OML 98-5, percent label values for shrimp receiving ^{15}N-glycine and ^{15}N-amino acid mix labeled feeds were not

Table 2.2: Percent incorporation of available label into shrimp muscle tissue.

OML	Day	Treatment	n	% incorporation
98-1	22	^{13}C-glycine	3	4.09 (0.33)
		^{15}N-glycine	3	6.41 (1.15)
		^{15}N-amino acid mix	3	9.36 (1.96)
98-5	20	^{15}N-glycine	3	8.32 (0.52)
		^{15}N-amino acid mix	3	8.17 (0.69)
		^{15}N/^{13}C-algal cells - N calculation	3	23.8 (0.9)
		^{15}N/^{13}C-algal cells - C calculation	3	19.2 (1.2)
99-3	18	^{15}N/^{13}C-algal cells - N calculation	2	27.2 (0.7)
		^{15}N/^{13}C-algal cells - C calculation	2	19.0 (0.5)

Numbers in ( ) are population standard deviations.
n = number of tanks in trial.

significantly different from the same additions in OML 98-1 (average values on day 20: 8.3 ± 0.5% and 8.2 ± 0.7%, respectively).

$^{15}N/^{13}C$-Algal Cell Labeled Feed

When the feed was labeled via the addition of $^{15}N/^{13}C$-algal cells, percent label values were more than double those found with similar amounts of crystalline labels. Average values calculated after approximately 3 weeks ranged from 19.0% to 27.3% (Table 2.2). Values calculated using nitrogen isotope values were significantly higher than those using carbon (one-way ANOVA; $p = 0.01$), indicating more complete assimilation of nitrogen and partial loss of carbon via respiration. Carbon values in OML 99-3 were similar to those in OML 98-5, while nitrogen values were significantly higher (one-way ANOVA combined with Tukey procedure; $p = 0.02$). All values for shrimp receiving feed with $^{15}N/^{13}C$-algal cells were statistically higher than those for shrimp receiving crystalline amino acids (one-way ANOVA combined with Tukey procedure; $p < 0.001$).

DISCUSSION

Nutrients

Nutrient concentrations were not different between control and treatment tanks for the same trial indicating that they had little influence on the isotope results presented here. Ammonia concentrations were well below the median lethal concentrations found

by Frías-Espericueta et al. (1999) for *P. vannamei* (70 to 110 mg/l ammonia-N depending on the length of exposure). Furthermore, the toxic unionized form of ammonia (NH_3) generally only represented a small percentage of the total TAN pool in these systems (personal communication, L. Conquest). Both TAN and $NO_2^- + NO_3^-$ levels were below median lethal concentrations determined for *Penaeus chinensis* after 72 hours of exposure (41.4 mg/l [approximately 2900 μM] for TAN and 117 mg/l [approximately 8300 μM] for NO_2^-; Chen et al., 1990).

General Isotopic Trends

The natural abundance $\delta^{13}C$ and $\delta^{15}N$ values in shrimp showed small increases in some experiments. The small increases in $\delta^{15}N$ for shrimp muscle and carapace with the growth of the shrimp (with the exception of muscle $\delta^{15}N$ for OML 99-3) contrasted with the $\delta^{13}C$ values, which were less uniform. The $\delta^{13}C$ had a weak increasing trend for muscle and carapace in OML 98-1 and weak decreasing trends for the final two experiments. Other studies, which examined the effects of growth and age on natural $\delta^{13}C$ and $\delta^{15}N$, have provided conflicting results regarding isotopic fractionation. Minagawa and Wada (1984) found no difference in $\delta^{15}N$ for tissue samples of two species of mussels as size and age increased. Frazer (1996) reported no change in $\delta^{13}C$ with increased length for larval krill and only a weak positive relationship between $\delta^{15}N$

and length. However, Rau et al. (1981) did see a significant increase in $\delta^{15}N$ in Dover sole with increased weight, which they attributed to changes in trophic status with age. Size differences may explain, in part, the statistically different starting isotopic values for muscle and carapace samples in OML 98-1 and OML 98-5, in spite of their similar feed labels. While the stocking weight of the shrimp was smaller in OML 98-5, these shrimp were statistically larger than those in the other trials on day 1. The starting $\delta^{15}N$ values shrimp from OML 99-3 had the lowest isotopic values due to lighter feeds.

Carapace, which is composed primarily of chitin, is significantly depleted in ^{15}N relative to muscle protein. This observation has been previously documented by DeNiro and Epstein (1981) who found that chitin from grasshoppers and milkweed bugs was depleted in ^{15}N relative to the diet. Additionally, Schimmelmann and DeNiro (1986) found that purified D-glucosamine hydrochloride from chitin in lobsters and ghost crabs was depleted in both $\delta^{15}N$ and $\delta^{13}C$ relative to muscle tissue. Unlike $\delta^{15}N$ values, there were no clear trends in $\delta^{13}C$ between muscle and carapace tissues in this study; however all differences were less than 1‰. This is similar to Schroeder's findings that *Macrobrachium rosenbergii* and penaeid carapace and muscle values were within 1‰ of each other (Schroeder, 1983a; Schroeder, 1983b).

Filtered particulate samples from controls and ^{15}N-labeled tanks in this study also increased in $\delta^{15}N$ over time but decreased in $\delta^{13}C$. Slight increases in nitrogen values may be due to slightly higher $\delta^{15}N$ in the feed and trophic enrichment within the shrimp. The $\delta^{13}C$ decline was likely the result of remnants of broken feed pellets appearing on the filters as the delta values matched or declined below values for the feed. Declines past the feed value may also be a result of senescence of algal cells and storage of lipids in the algal fraction near the end of the experiment.

Feeding Experiments: Crystalline Amino Acids

Carapace showed a more rapid response to the new food label than did muscle tissue in all of the label addition experiments. This response was also detected in similar diet switching experiment on *Penaeus setiferus* (Parker et al., 1991), and is likely due to rapid replacement of carapace after molting. The limited incorporation of the labeled free amino acid carbon and nitrogen by shrimp in these experiments is assumed due to partial dissolution of the label during feeding and soaking in the ponds. Reduced labeling may also be due to the fact that the amino acids used were nonessential for the shrimp, but this is unlikely. Glycine is not considered an essential amino acid for shrimp (Akiyama et al., 1992; Goddard, 1996), but it is a precursor in transamination as well as a protein constituent. Additionally, similar low incorporation results for the amino acid

mix (approximately 52% of the mixture were essential amino acids) further supports dissolution as the cause of low label incorporation.

The increase in the isotopic label by the SPOM samples could be a result of the appearance of broken feed pellets on the filter or uptake of the label by pond algae. In the OMLs, aeration provides for constant mixing of the materials in the tank, and the uneaten pellets could become part of the suspended material. Given that the change in SPOM $\delta^{15}N$ and $\delta^{13}C$ signatures was gradual in that values nearly matched the labeled feed only after at least 2 weeks of feeding labeled feeds, it is also likely that recycling of the isotopic label within the tanks is a contributing factor to isotopic increases. The outdoor mesocosm tanks are designed to naturally mineralize and oxidize reduced nitrogen liberated during the process of shrimp aquaculture.

Feeding Experiments: Proteinaceous Compounds in Labeled Algal Cells

The amount of label reflected in the muscle tissue was higher when the label was presented in particulate form (algal cells) rather than as crystalline amino acids. The higher incorporation for nitrogen label versus carbon label for the doubly labeled algal cells indicates mineralization and re-uptake of the nitrogen fraction. Once metabolized, most of the carbon label is lost to the carbon dioxide pool whereas the nitrogen is re-assimilated and available as shrimp food. Some of the observed increase

(~3‰) may be due to the higher metabolic fractionation of nitrogen by the shrimp. The appearance of the label in the SPOM fraction again suggests broken feed pellets on the filter.

CONCLUSIONS

Results from the initial feeding experiments showed that stable isotopic labels are easily tracked into shrimp tissues. Addition of the label as a crystalline compound did not work well. When the label was added in a proteinaceous form (algal cells), shrimp incorporated a much larger fraction of the label. Appearance of the label in SPOM indicated that there may be important recycling pathways. Labeling endeavors should attempt to label ingredients that are not water soluble and analogous to the bulk feed components in order to measure representative uptake and assimilation rates. This technique offers powerful insight into feed utilization efficiency

CHAPTER 3 – POLLOCK STICKWATER AS A FEEDING STIMULANT IN ZERO-WATER EXCHANGE SHRIMP CULTURE

INTRODUCTION

Shrimp are known to utilize chemical stimuli for a variety of behaviors including attraction to feed and stimulation of feeding (Carr and Derby, 1986; Dall et al., 1990; Hartati and Briggs, 1993; Lee and Meyers, 1996). Multiple components, primarily low molecular weight compounds such as amino acids, nucleosides, nucleotides, quaternary ammonium compounds, and organic acids (Carr and Derby, 1986), have been found to act as attractants for shrimp. For instance, Costa-Pierce and Laws (1985) found trimethylammonium hydrochloride increased ingestion rates in the prawn *Macrobrachium rosenbergii* when added to their feed. Many of the early studies on chemoattraction specifically noted responses by shrimp to the presence of amino acids in the water (Carr and Derby, 1986; Carr et al., 1984; Hartati and Briggs, 1993; Hindley, 1975; Zimmer-Faust, 1987). Zimmer-Faust (1987) examined the amino acids taurine, glycine, and glutamic acid on six different species of crustacean and found the animals searching out the source of the amino acids. He argued that amino acid release during the decomposition of animals attracts crustaceans. The concomitant release of

ammonia, however, was suppressive. He concluded that the ratio of free amino acid to ammonia signaled the shrimp of the nutritional quality of the dead organism.

Continued research indicated that while individual amino acids did act as attractants to shrimp, they were not as effective as more complex mixtures (Mackie and Shelton, 1972; Carr et al., 1984). Mackie and Shelton (1972) found that a mixture of extracts from squid was more effective as an attractant for the lobster *Homarus gammarus* than were the individual components of the extract. Carr et al. (1984) found that crab extracts elicited a greater feeding response in *Palaemonetes pugio* than an amino acid mix plus betaine, which had previously been determined effective for this species. As they experimented further to duplicate the effect of the crab extract, they found that the amino acid mixture along with purines and purine nucleotides, homarine, trimethylamine oxide, and lactic acid caused the shrimp to behave similarly.

Given the information found from the above studies, current feed formulations for shrimp aquaculture often include squid meal or mussel flesh as an attractant. Yet, there is limited information on amounts of attractant and stimulant necessary for commercially important species as well as the means to present them to the shrimp (Lee and Meyers, 1996). The addition of these substances to aquaculture feed formulations

is, nevertheless, important in terms of reducing costs and production of waste materials (Lee and Meyers, 1996; Lee and Meyers, 1997).

The fishing industry in Alaska accounts for approximately 45% of the nation's catch (Rathbone and Babbitt, 2000). Coincident with this, the Alaskan fishing industry produces large amounts of by-product waste, with over 1 million tons of in 2000 (Bechtel and Crapo, 2002). Given the quantities involved, efforts are underway to find alternate uses for this material. One of the by-products of fish processing is stickwater. Stickwater is produced by the cooking of the waste materials during fishmeal processing and is separated when the material is pressed. The liquids that are pressed from the meal are separated into oil and stickwater (the remaining solubles) through centrifugation (Rathbone and Babbitt, 2000). Stickwater is high in suspended and dissolved proteins (Gaudé, 1994), and is often added to and dried with the fishmeal. Its composition also indicates that it might be useful as an attractant or decrease the cost of attractants in shrimp feed.

One of the important distinctions to make when discussing chemoattraction in any species is the difference between attraction and feeding stimulation (Lee and Meyers, 1996; Lee and Meyers, 1997). A shrimp may find food due to chemical signals, but this does not mean it will eat that food. This distinction is usually determined in

carefully controlled laboratory experiments in which the shrimp response to its food is monitored (Costero and Meyers, 1993). These results may or may not adequately describe the shrimp response under normal culture conditions due to the complexity of the pond environment.

Stable isotope experiments with labeled feeds were conducted to assess the stimulant nature of pollock stickwater by comparing the uptake of several feeds under normal culture conditions. Formulated feed, with and without attractants/stimulants, and tank natural production all contribute to shrimp growth. The use of stable isotopic tracers allows the assessment of growth in response to specific feed constituents, without changing growth conditions in other respects. This chapter discusses initial research using stable isotopic tracers to determine whether pollock stickwater, produced as a by-product of Alaskan fisheries, is effective as a feeding stimulant for cultures of *Litopenaeus vannamei*.

MATERIALS AND METHODS

Shrimp rearing and feeding

The investigation of stickwater as a feeding stimulant was undertaken in both indoor and outdoor trials at the Oceanic Institute (OI). The indoor trials were performed to assess the shrimp response without the influence of natural tank phytoplankton

production. Outdoor trials were done to investigate response under normal culture conditions.

Indoor

For the indoor trials, 15 glass aquaria (52 L) were stocked with 36 shrimp each on 5 January 2001. The mean starting weight of the shrimp was 0.57 g. These were flow-through systems with complete seawater exchange every hour. Uneaten feed, feces, molts and dead shrimp were removed every morning. Shrimp were fed 8 times throughout a 24-hour period by automatic feeders. Wet weights were determined bi-weekly during the trial. The shrimp were maintained on a 12-hour day/night cycle throughout.

Outdoor

Fifteen outdoor tanks (1300 L) were stocked with 0.82 g shrimp (mean weight) at a density of 100 shrimp per tank on 4 January 2001. The outdoor systems were zero-water exchange with freshwater additions made only to counteract evaporative losses. Shrimp were fed on a 24-hour basis using a feeding belt. Wet weights were determined bi-weekly throughout the trial. Water quality was monitored weekly by the OI staff for nitrate + nitrite and ammonia. Due to some unexplained mortality and concerns over the nighttime winter temperatures, clear covers were placed over the tanks at all times after

day 10 in order to maintain a temperature of approximately 25°C. After this time, shrimp mortality was minimal.

Treatments

Shrimp in the control experiments for indoor trials were fed a reference feed which had 40% crude protein and 8.5% crude lipid (see Appendix D for feed formulations). Outdoor shrimp received feed with 30% crude protein and 8% crude lipid. The control feeds contained squid liver powder (SLP) at 2.5% as the traditional attractant. In both the indoor and outdoor trials, the treatments consisted of:

A. feed with squid liver powder (2.5%)
B. feed without any added attractant
C. feed with squid liver powder replaced by 1% pollock stickwater (freeze-dried solid)
D. feed with squid liver powder replaced by 2.5% pollock stickwater

Each of the above feeds utilized a plant protein source ("green feed") rather than the standard fishmeal. Aquaculturists have been trying to reduce the amounts of fishmeal in formulated feeds due to the financial and ecological costs (Masser, 2000). Good growth on plant-based feeds by the shrimp would likely be a result of the added attractant/stimulant. Additionally, shrimp in the indoor trial also received a control feed (with squid liver powder) which had a fishmeal protein source. Isotopic label was added in the form of ^{15}N-algal *Agminelum quadriplicata* cells (5g/19 kg feed; Cambridge

Scientific NLM-2162; 96-99% ^{15}N atom enriched) to each of these feeds. Pollock stickwater (provided by the Kodiak Fishmeal Company in Kodiak, Alaska; October 2000) was freeze-dried and added to the feed at 1% and 2.5% dry weight.

Sampling

All treatments were assigned randomly to three tanks each. Three shrimp were sampled biweekly from the tanks throughout the trial and frozen for subsequent analysis. Additionally, in outdoor trials, samples of tank suspended particulate organic matter (SPOM) were taken at the same time as the shrimp by filtering 10 to 20 ml of water through 25 mm GF/F filters. For indoor trials, initial shrimp isotopic values were determined for shrimp sampled on 12 January 2001. After these shrimp were removed, the remaining shrimp were switched to the diet containing the isotopic label until they were harvested on 2 February. In outdoor trials, zero-time shrimp samples were taken on 10 January 2001. Labeled feed was given to the shrimp after zero-time SPOM samples were taken on 11 January and continued until the end of the trial on 1 February. Shrimp were fed continuously using a belt feeder, and their daily ration gradually decreased from 8 to 5.5% of their body weight in the ICLs and 8 to 7% in the OMLs.

Sample Preparation and Analysis

Two shrimp from two of the three tanks per treatment were prepared for isotopic

analysis. Carapace and filter samples were prepared as described in Chapter 2 with overnight acidification and drying prior to mass spectrometer analysis. Muscle samples were freeze-dried at the Oceanic Institute for 12-16 hours with the exception of the last sampling day (1 February for the outdoor trials and 2 February for the indoor trials). These samples were frozen and sent to UAF for similar preparation. Dried muscle samples and feed samples were ground into a fine homogenous powder before analysis on an elemental analyzer coupled to a Finnigan Delta Plus mass spectrometer.

Calculation of Percent Label in Muscle

The percent of the label appearing in the shrimp muscle tissue was determined in order to compare feed utilization under conditions in which the $\delta^{15}N$ values of the feeds were different. This was done by comparing the amount of ^{15}N in shrimp muscle tissue to the amount added via labeled feed as described in Chapter 2. See Appendix C for a sample calculation.

Statistics

One-way ANOVA was used to compare growth and efficiency measures as well as percent label for the different feeding trials. Shrimp sampled from tanks were averaged for one tank value that was used as the repeated measure in ANOVA. FCR was log transformed prior to statistical analysis. A Tukey test for multiple comparisons

was used in association with the one-way ANOVA to examine specific means for significant differences. Nutrient concentrations for the tanks in the outdoor trials were compared using a general linear model (GLM) analysis in which day and treatment were used to model concentrations (Minitab version 8.21 for Macintosh). Values were reported as mean ± an estimate of population standard deviation.

RESULTS

Growth and Efficiency Measures

The growth and efficiency measures for the indoor and outdoor trials are given in Tables 3.1 and 3.2.

Indoor

Shrimp in the indoor tanks grew on average 0.2 ± 0.03 g/wk (mean ± population standard deviation). Survival for these tanks was high (87 – 100%), and the FCR values ranged from 2.1 to 3.3 g feed/g wet weight of the shrimp. Final shrimp weights for this trial ranged from 1.0 g to 1.4 g. Shrimp fed the fish based protein feed had significantly higher weekly growth than shrimp fed 2.5% stickwater amended feed (one-way ANOVA combined with Tukey procedure; $p = 0.03$), with average growth of 0.2 g/wk as compared to 0.1 g/wk. Furthermore, shrimp fed the fishmeal had a higher SGR (3.2 ± 0.1%/day) than both SLP (2.6 ± 0.2%/day) and 2.5% stickwater feeds (2.4 ± 0.3%/day;

Table 3.1: Shrimp growth and efficiency measures for indoor feeding attractant/stimulant experiments.

Treatment	n	Initial Wt (g)	Final Wt (g)	Weight Gain (%)	Growth (g/wk)	FCR (g feed/g wet wt shrimp)	SGR (%/day)	Survival (%)
Green feed + SLP	2	0.59 (0.00)	1.23 (0.05)	109 (8)	0.16 (0.01)	2.70 (0.21)	2.65 (0.14)	95.0 (2.4)
Green feed, no SLP	2	0.56 (0.00)	1.16 (0.10)	107 (16)	0.15 (0.02)	2.80 (0.34)	2.57 (0.29)	95.0 (2.4)
Green feed + 1% stickwater	2	0.56 (0.00)	1.31 (0.12)	132 (20)	0.18 (0.03)	2.33 (0.22)	2.99 (0.29)	96.7 (4.7)
Green feed + 2.5% stickwater	2	0.56 (0.00)	1.10 (0.08)	95.5 (13.9)	0.14 (0.02)	3.04 (0.40)	2.37 (0.25)	93.3 (0.0)
Control ICL feed	2	0.57 (0.01)	1.39 (0.04)	146 (11)	0.21 (0.01)	2.18 (0.08)	3.19 (0.14)	93.3 (0.0)

Numbers in ( ) represent population standard deviations

n = number of tanks for each treatment

Table 3.2: Shrimp growth and efficiency measures for outdoor feeding attractant/stimulant experiments.

Treatment	n	Initial Wt (g)	Final Wt (g)	Weight Gain (%)	Growth (g/wk)	FCR (g feed/g wet wt shrimp)	SGR (%/day)	Survival (%)
Green feed + SLP	2	0.83 (0.02)	3.03 (0.06)	265 (1)	0.55 (0.01)	1.42 (0.08)	1.85 (0.00)	94.3 (8.1)
Green feed, no SLP	2	0.80 (0.01)	2.92 (0.03)	263 (2)	0.53 (0.00)	1.43 (0.03)	1.84 (0.01)	97.5 (0.0)
Green feed + 1% stickwater	2	0.84 (0.01)	3.21 (0.46)	283 (61)	0.59 (0.12)	16.1 (15.1)	1.91 (0.23)	28.3 (4.2)
Green feed + 2.5% stickwater	2	0.83 (0.01)	3.28 (0.25)	298 (35)	0.61 (0.06)	2.20 (1.29)	1.97 (0.13)	72.2 (37.6)

Numbers in ( ) represent population standard deviations

n = number of tanks for each treatment

one-way ANOVA combined with Tukey procedure; $p = 0.02$).

Outdoor

Shrimp in the outdoor trial grew an average of 0.6 ± 0.1 g/wk to a final weight of approximately 3.1 ± 0.3 g. Growth rates for shrimp maintained in the outdoor tanks were significantly higher than for those in indoor trials (one-way ANOVA; $p < 0.001$). Shrimp FCR (16.1 ± 15.1 g feed/g wet weight shrimp) and mortality ($28.3 \pm 4.2\%$ survival) was highest for tanks receiving 1% stickwater. However, there were no statistically significant differences in weight, SGR, FCR, or survival for these trials.

Nutrients

The nutrient profiles for the outdoor treatments are presented in Figure 3.1. NO_3^- + NO_2^- levels decreased during the experiment from 68.4 ± 19.6 μM six days prior to the addition of the labeled feeds to approximately 1 μM by the end of the experiment. TAN concentrations were generally below the limit of detection but reached as high as 80 μM in one of the tanks fed 1% stickwater amended feed. Nevertheless, these values are not considered toxic to the shrimp (Alcaraz et al., 1999b). GLM analysis indicated that the differences in nutrient concentrations over the period of the experiment could be accounted for by day alone. Treatment did not cause variations in nutrient concentration.

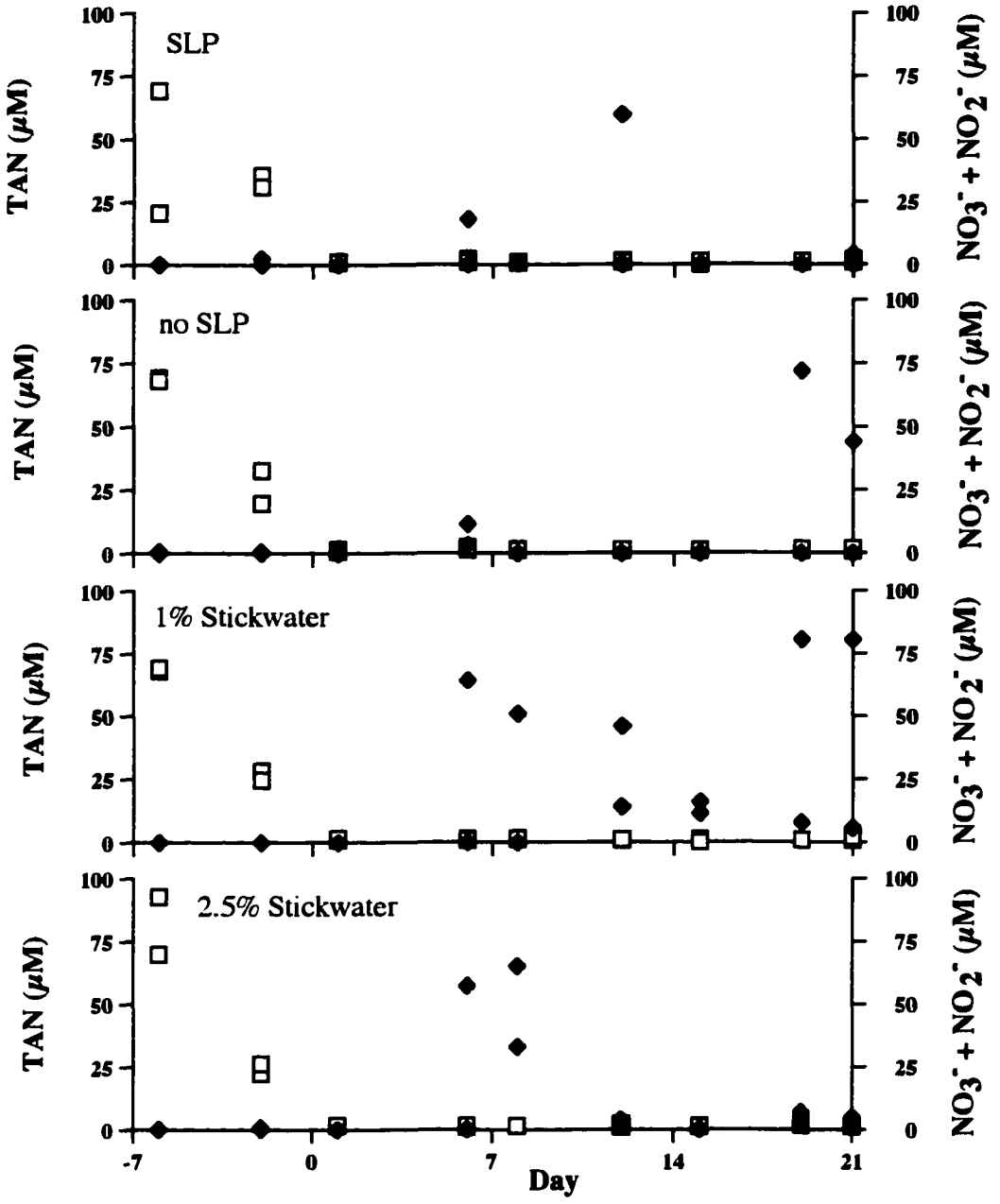

Figure 3.1: Nutrient profiles for outdoor attractant/stimulant trials. SLP = feed with squid liver powder as an attractant, no SLP = feed without an attractant, stickwater = pollock processing waste used in place of SLP at two different concentrations. □ $NO_3^- + NO_2^-$; ◆ TAN.

Isotopic Results

Indoor

$\delta^{15}N$ and $\delta^{13}C$ results for the indoor trials are presented in Figures 3.2 to 3.6. The $\delta^{15}N$ values for muscle and carapace on day 1 prior to the addition of the labeled feed were similar. Additionally, carapace values are initially around 3‰ lower than muscle (as noted in Chapter 2). When labeled feed was given to the shrimp, their isotope ratios changed in response to the new signature for the feed. Due to the rapid growth of the shrimp and the molting that accompanies it, carapace values responded more quickly to the ^{15}N enrichment of the feed than did muscle values. The $\delta^{15}N$ values increased from a day 1 mean of 9.5 ± 0.2‰ for all tanks (n = 10 tanks, ± standard deviation) to 24.4 ± 1.5‰ (n = 8) for shrimp receiving the amended 'green feeds' (Figures 3.2 to 3.5) and 29.8 ± 0.3‰ (n = 2) for shrimp receiving the standard fishmeal based feed (Figure 3.6). Carapace $\delta^{15}N$ values increased in a similar fashion from 5.5 ± 0.4‰ on day 1 to 28.8 ± 2.4‰ for the 'green' feeds and 36.6 ± 0.2‰ for the fishmeal tanks by day 22.

Carbon values for both muscle and carapace in all treatments showed similar trends over the course of the experiment. $\delta^{13}C$ values were not expected to change and showed only minor variations, with muscle values of –20.2 ± 0.2‰ to –21.0 ± 0.4‰ from day 1 to day 22 (n = 10 tanks). Carapace values were –19.3 ± 0.3‰ and –19.6 ± 0.6‰ over the same period.

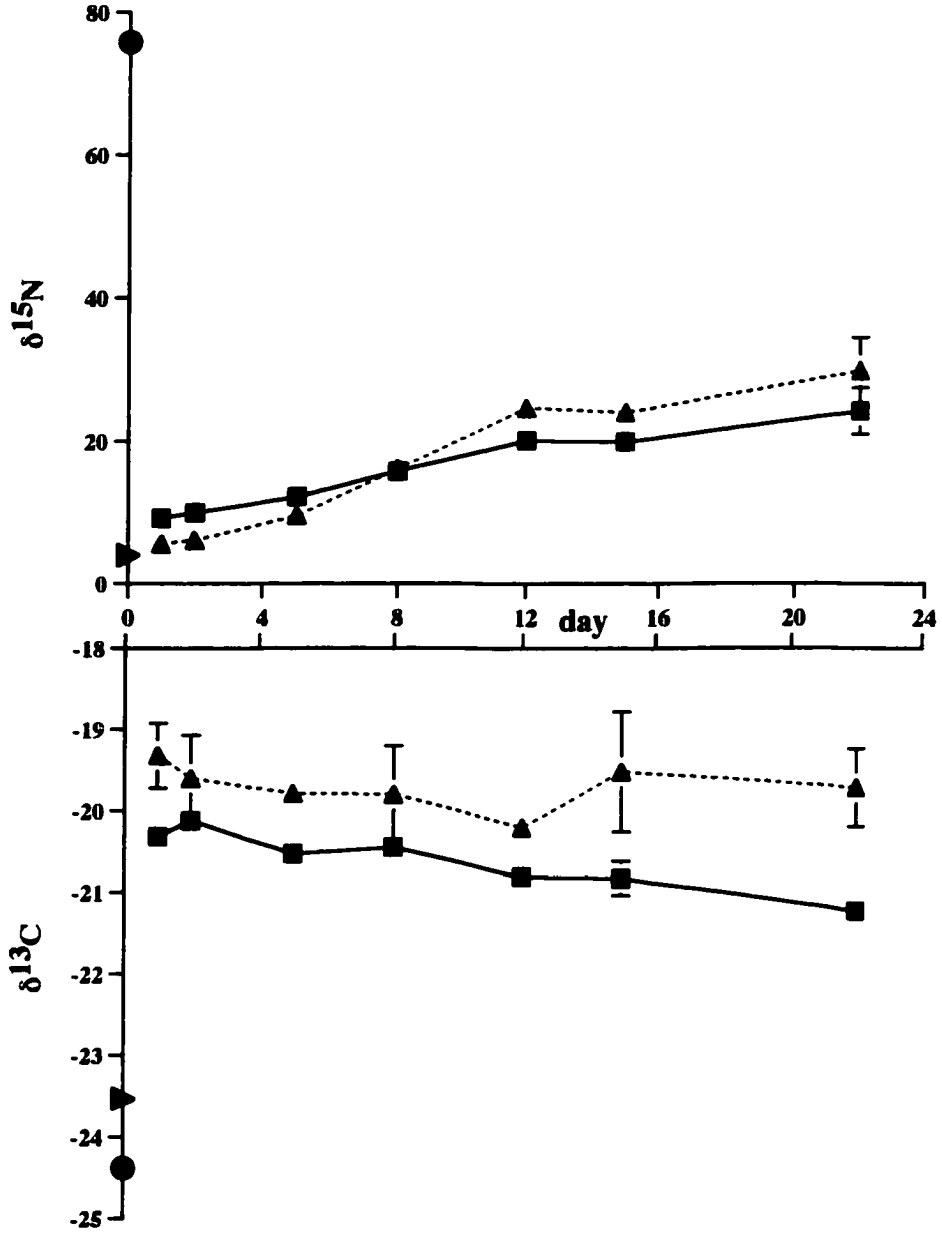

Figure 3.2: Average $\delta^{15}N$ and $\delta^{13}C$ values for indoor tanks receiving plant-based 'green feed' + squid liver powder as an attractant. Error bars represent population standard deviation for two tanks. ■ shrimp muscle; ▲ shrimp carapace; ◗ initial feed; • labeled feed.

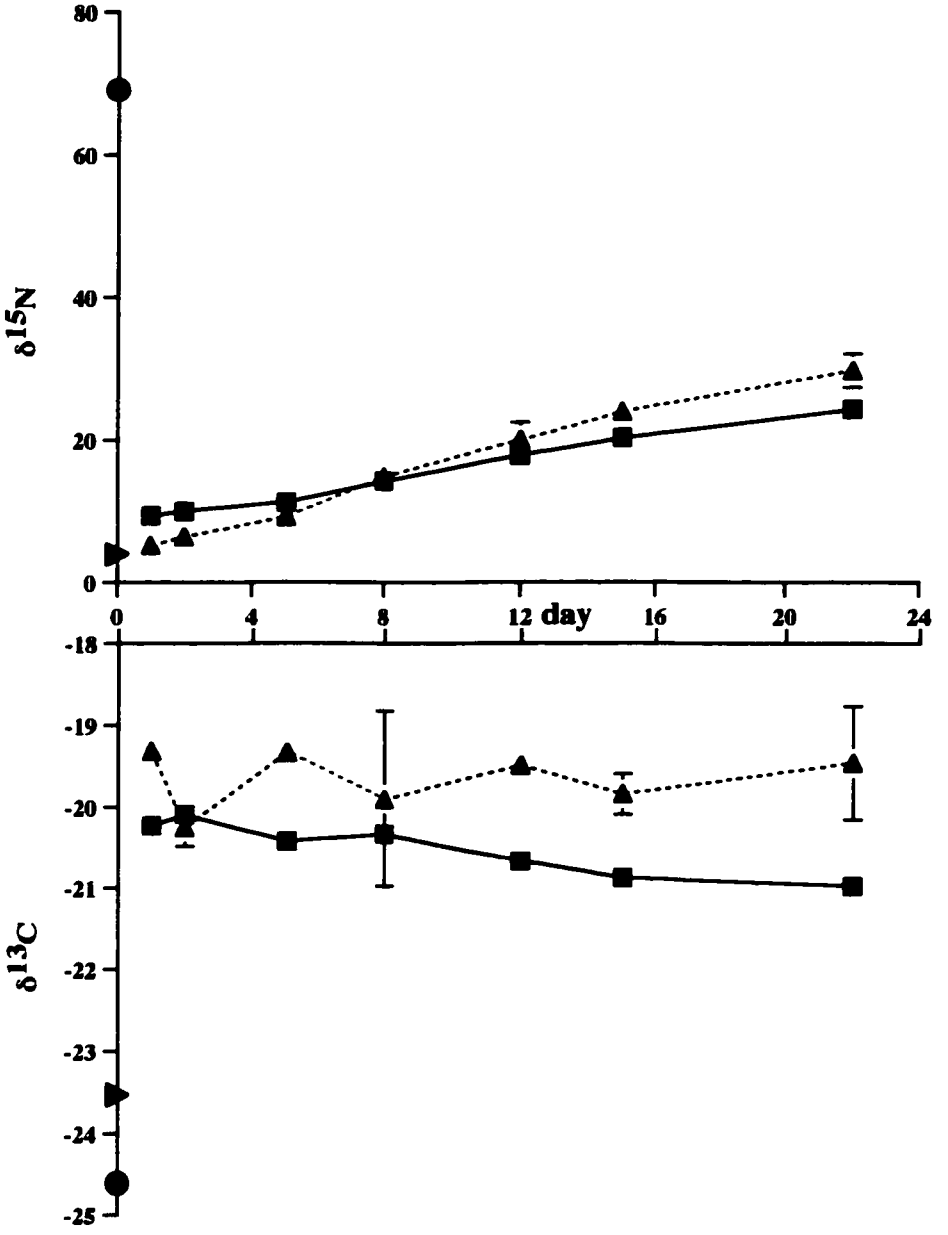

Figure 3.3: Average $\delta^{15}N$ and $\delta^{13}C$ values for indoor tanks receiving plant-based 'green' feed without added attractant. Error bars represent population standard deviation for two tanks. ■ shrimp muscle; ▲ shrimp carapace; ▶ initial feed; • labeled feed.

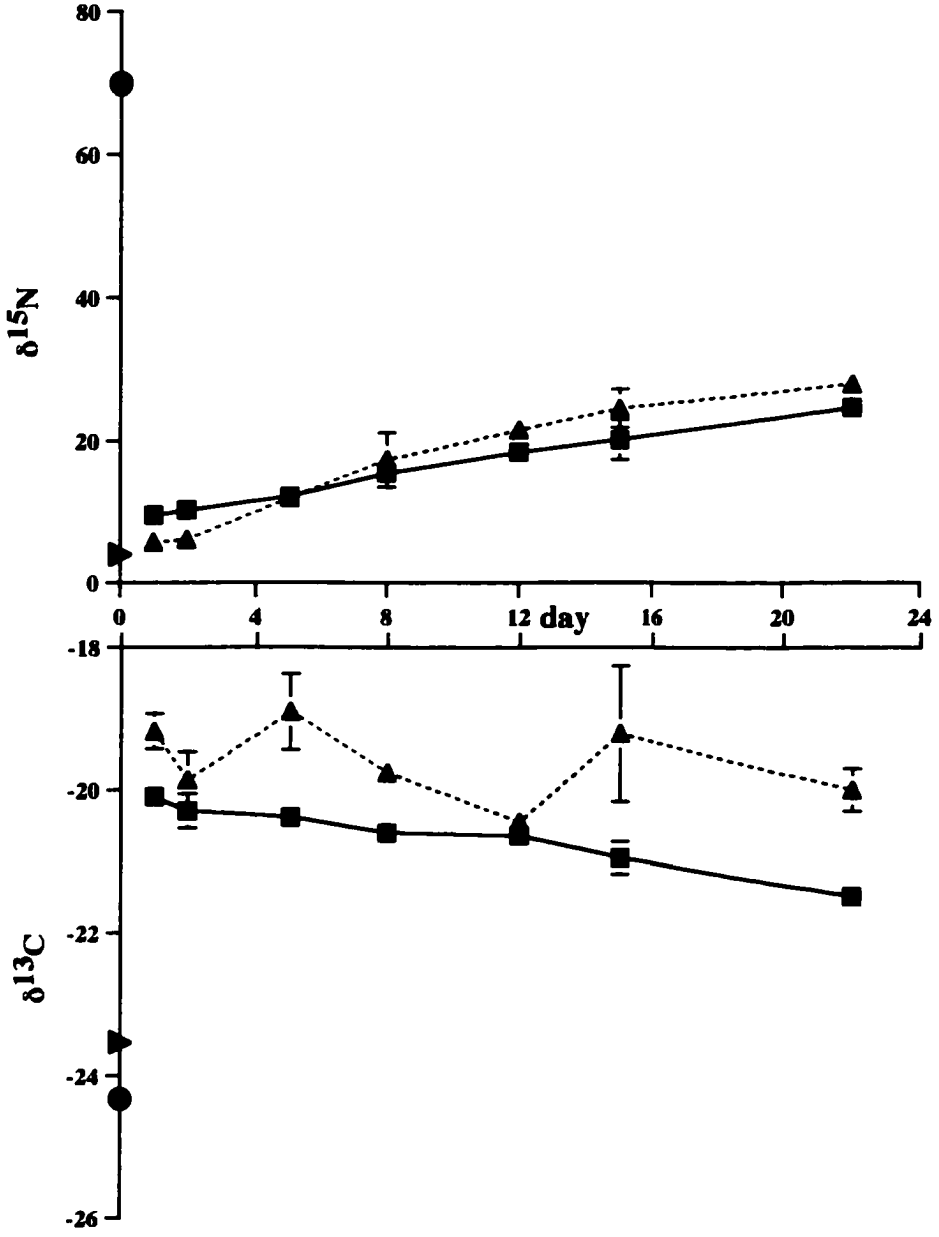

Figure 3.4: Average $\delta^{15}N$ and $\delta^{13}C$ values for indoor tanks receiving plant-based 'green' feed + 1% pollock stickwater as a feeding stimulant. Error bars represent population standard deviation for two tanks. ■ shrimp muscle; ▲ shrimp carapace; ◗ initial feed; • labeled feed.

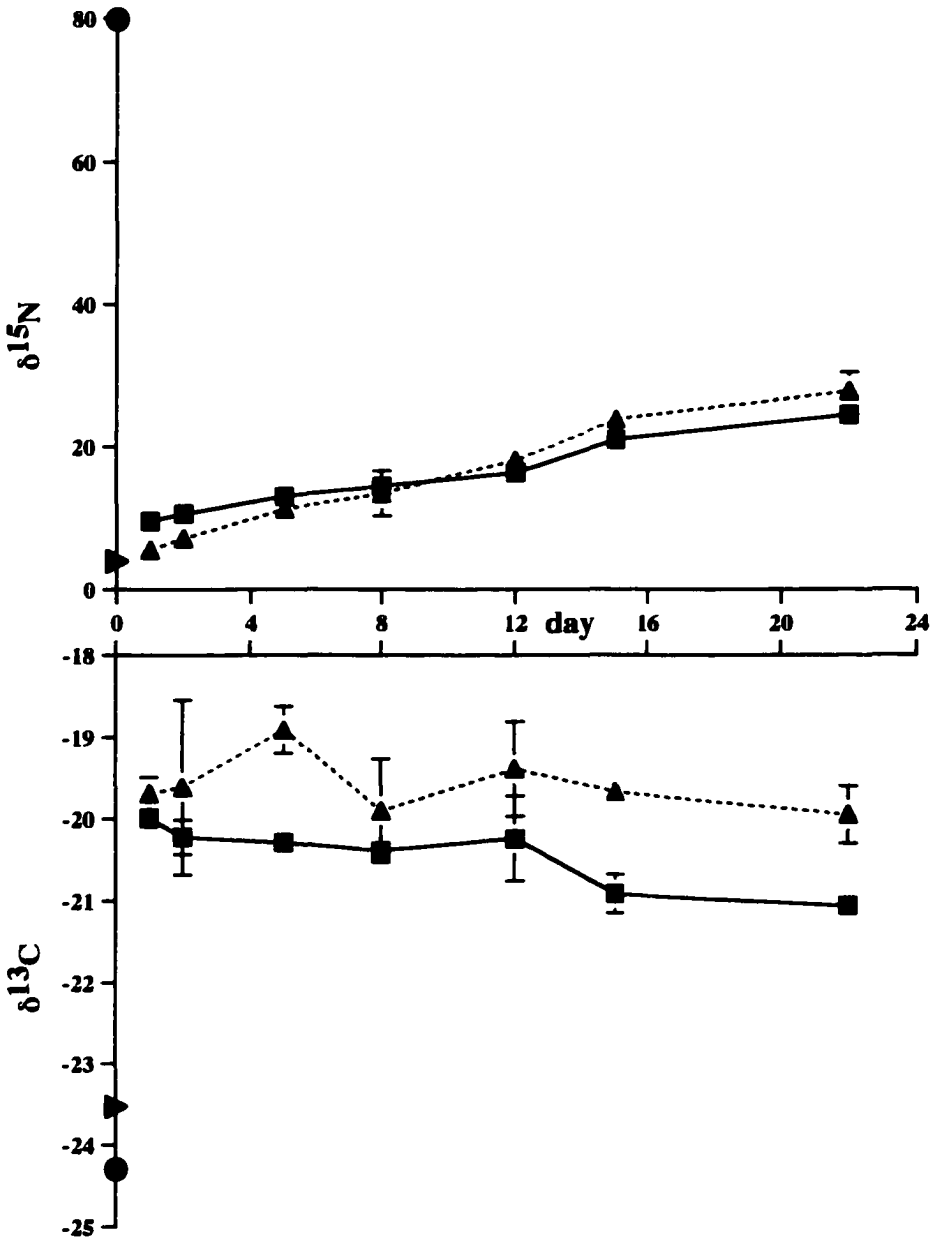

Figure 3.5: Average $\delta^{15}N$ and $\delta^{13}C$ values for indoor tanks receiving plant-based 'green' feed + 2.5% pollock stickwater as a feeding stimulant. Error bars represent population standard deviation for two tanks. ■ shrimp muscle; ▲ shrimp carapace; ▸ initial feed; • labeled feed.

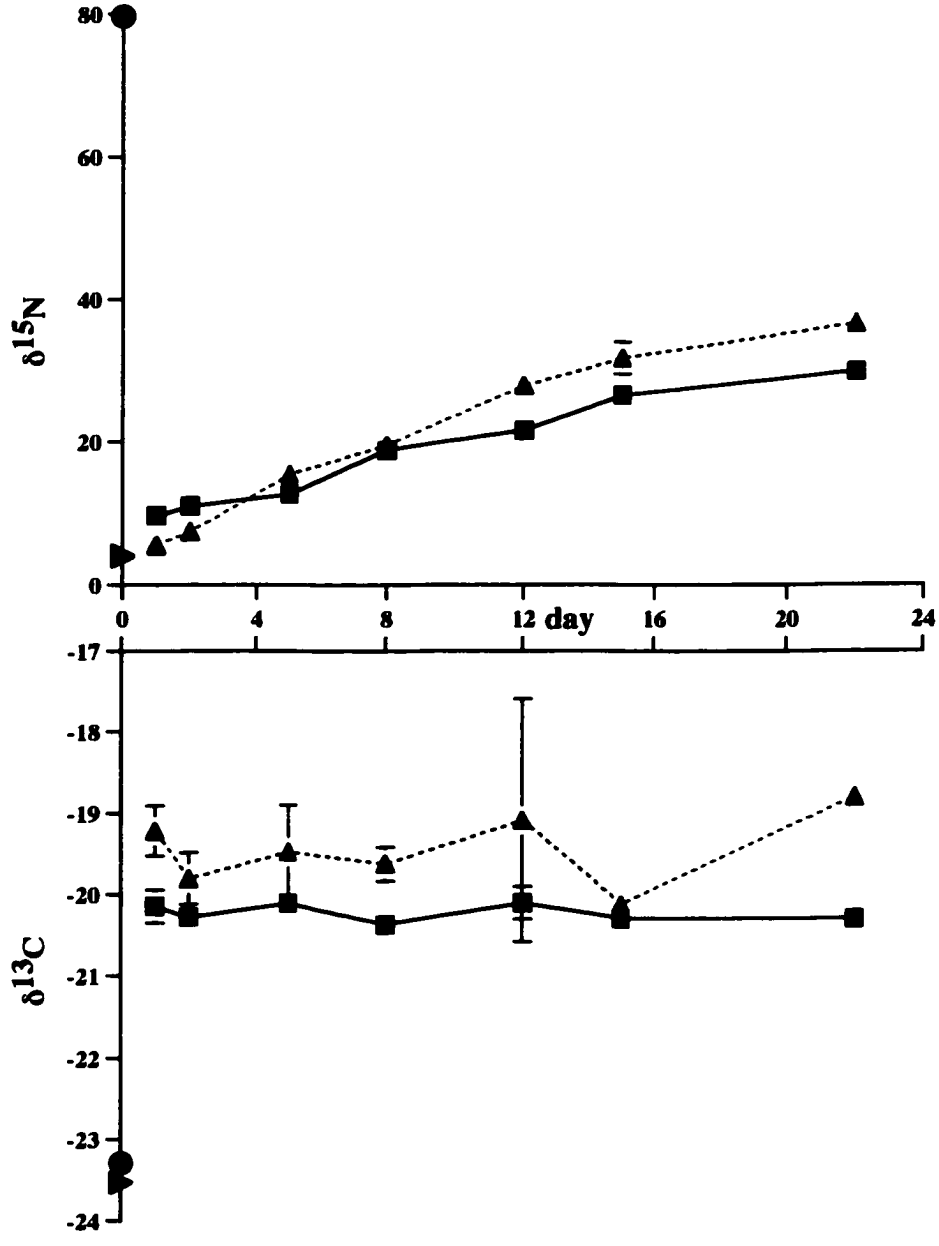

Figure 3.6: Average $\delta^{15}N$ and $\delta^{13}C$ values for indoor tanks receiving standard fishmeal based feed with squid liver powder as an attractant. Error bars represent population standard deviation for two tanks. ■ shrimp muscle; ▲ shrimp carapace; ◗ initial feed; • labeled feed.

Outdoor

Shrimp in the outdoor tank treatments showed greater growth responses to the labeled feed than those in indoor tanks. These results are given in Figures 3.7 to 3.10. Shrimp in the outdoor trials had day 1 muscle natural abundance $\delta^{15}N$ values that were significantly depleted compared to shrimp in the indoor tanks. Average starting $\delta^{15}N$ values for muscle were 8.6 ± 0.5‰, 8.8 ± 0.2‰, 8.4 ± 0.03‰, and 8.3 ± 0.04‰ for tanks receiving SLP, no attractant, 1% stickwater, and 2.5% stickwater respectively (n = 2 tanks).

Muscle $\delta^{15}N$ for the shrimp receiving SLP feed (feed $\delta^{15}N$ = 108.5‰) increased over the study period to 49.4 ± 2.2‰ by day 22 (Figure 3.7). Over that same period, carapace $\delta^{15}N$ increased sharply from approximately 5‰ to 58.0 ± 6.8‰. Additionally, samples of the tank SPOM showed enrichment in $\delta^{15}N$ from an average of 8.9 ± 0.5‰ to values of 92.8 ± 3.5‰ by the end of the experiment, indicating either the appearance of broken feed pellets on the filters or rapid recycling of mineralized nitrogen into phytoplankton biomass.

When shrimp were fed a feed without an added attractant/stimulant, their muscle and carapace $\delta^{15}N$ increased to average values of 45.8 ± 0.1‰ and 52.2 ± 0.4‰ for muscle and carapace, respectively, on day 22 as compared to the feed label of 103.9‰

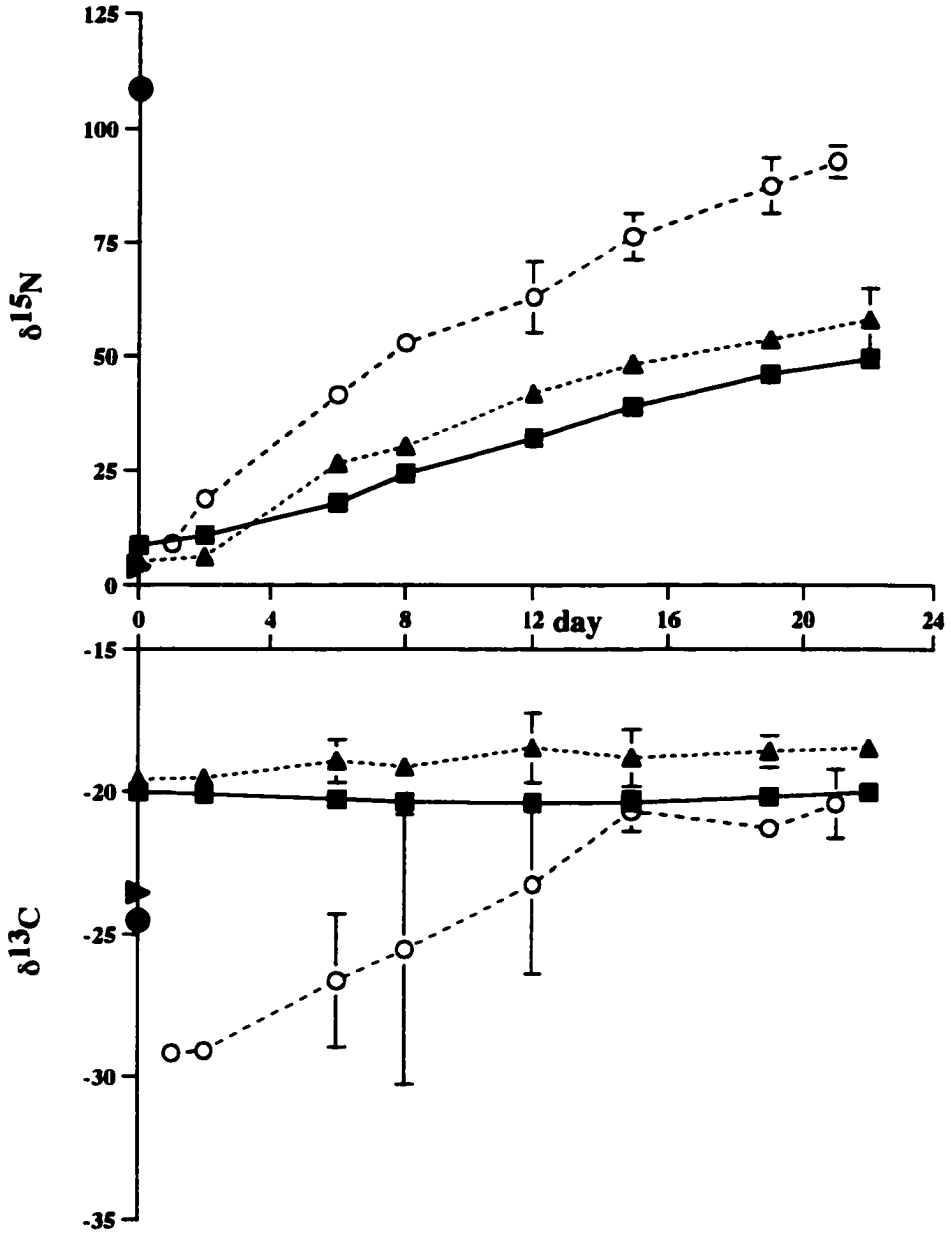

Figure 3.7: Average $\delta^{15}N$ and $\delta^{13}C$ values for outdoor tanks receiving plant-based 'green feed' + squid liver powder as an attractant. Error bars represent population standard deviation for two tanks. ■ shrimp muscle; ▲ shrimp carapace; O SPOM; ▸ initial feed; • labeled feed.

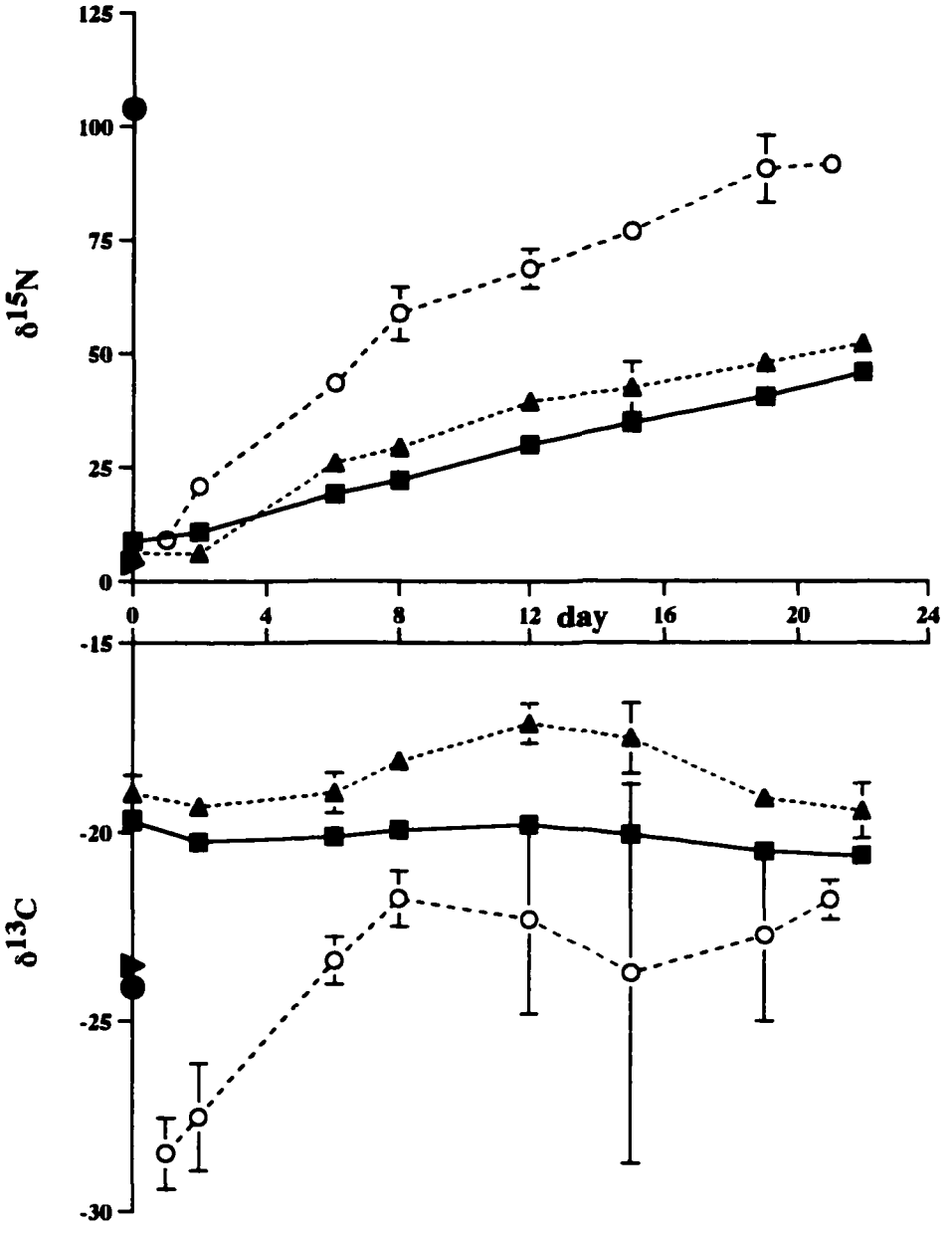

Figure 3.8: Average $\delta^{15}N$ and $\delta^{13}C$ values for outdoor tanks receiving plant-based 'green' feed without added attractant. Error bars represent population standard deviation for two tanks. ■ shrimp muscle; ▲ shrimp carapace; O SPOM; ▸ initial feed; • labeled feed.

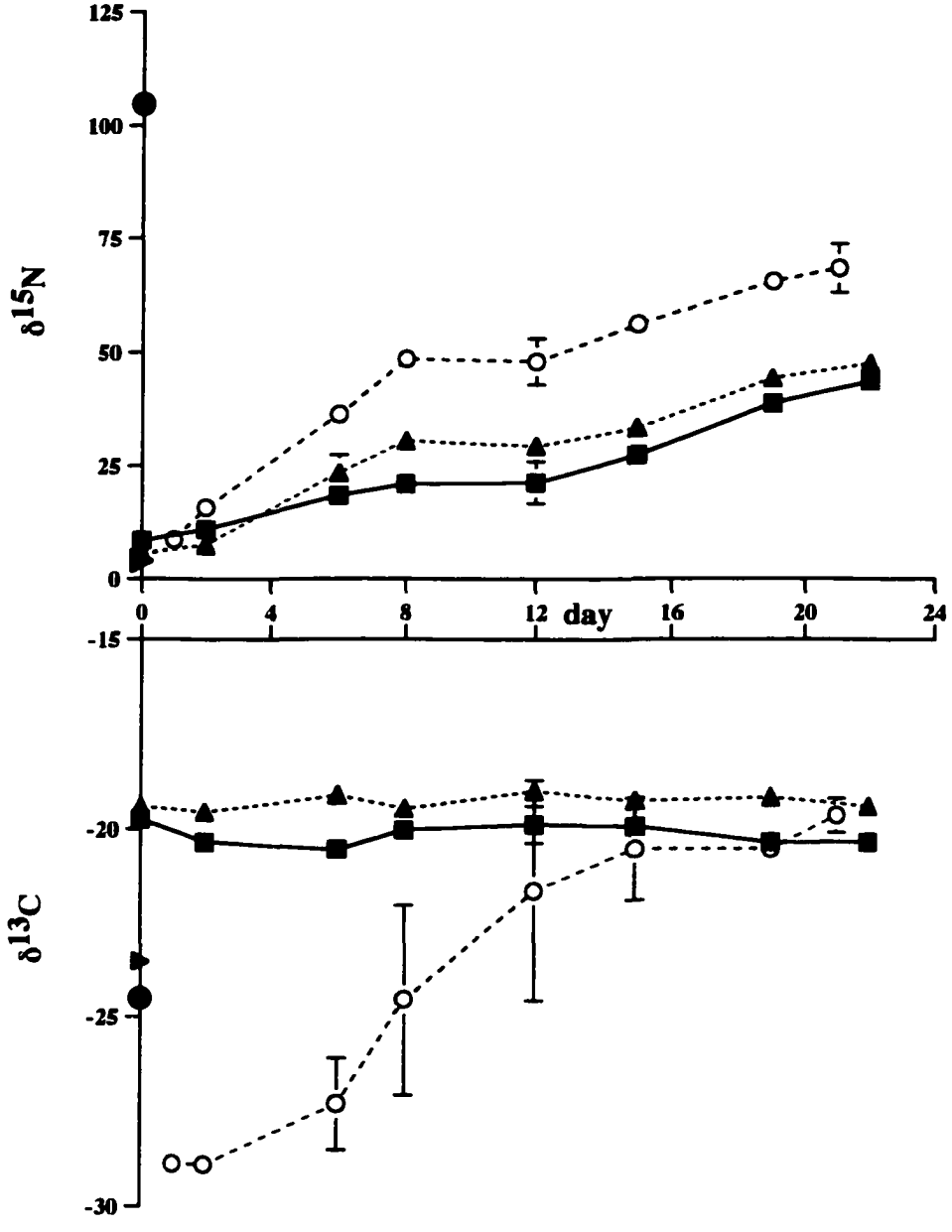

Figure 3.9: Average $\delta^{15}N$ and $\delta^{13}C$ values for outdoor tanks receiving plant-based 'green' feed + 1% pollock stickwater as a feeding stimulant. Error bars represent population standard deviation for two tanks. ■ shrimp muscle; ▲ shrimp carapace; O SPOM; ▶ initial feed; • labeled feed.

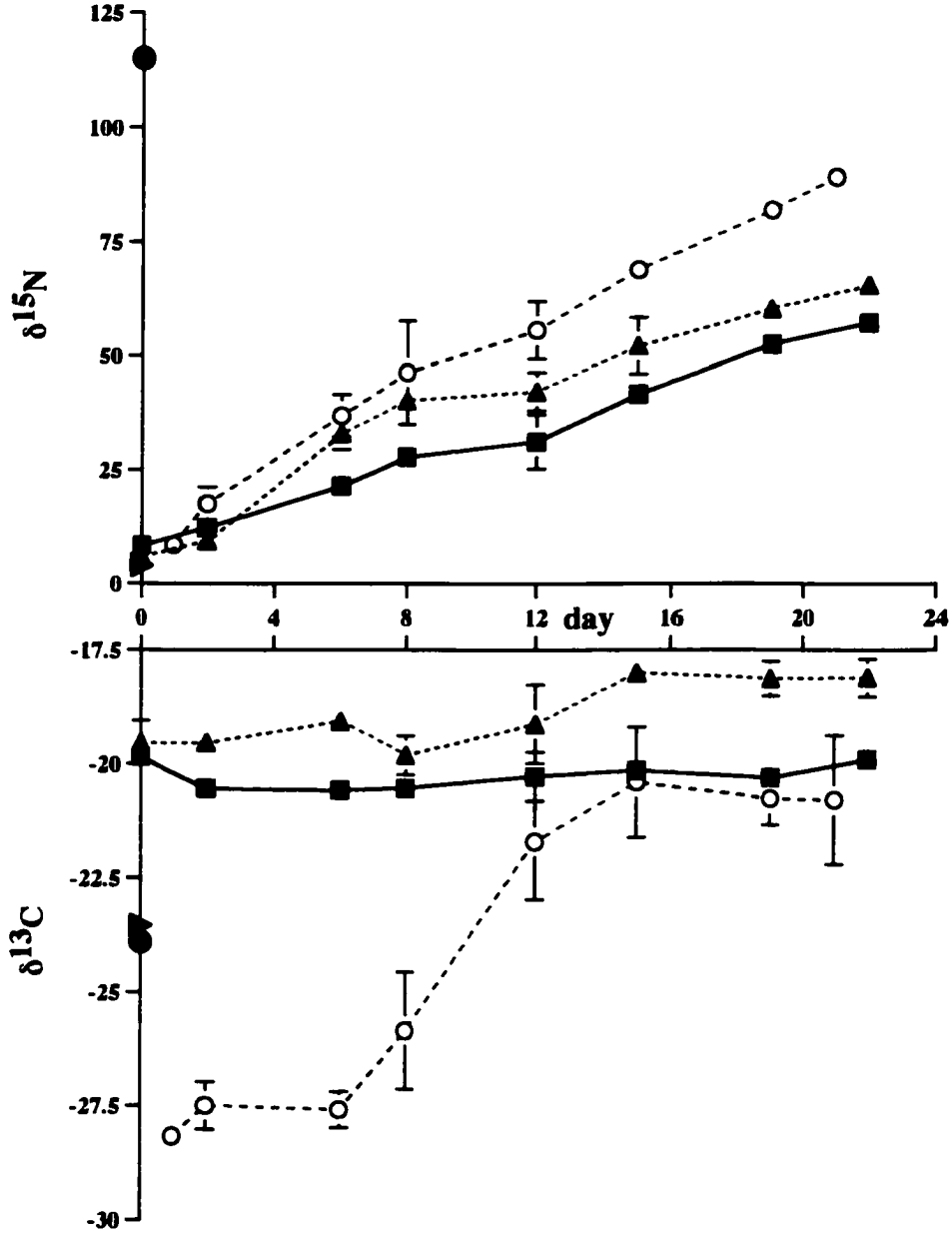

Figure 3.10: Average $\delta^{15}N$ and $\delta^{13}C$ values for outdoor tanks receiving plant-based 'green' feed + 2.5% pollock stickwater as a feeding stimulant. Error bars represent population standard deviation for two tanks. ■ shrimp muscle; ▲ shrimp carapace; O SPOM; ▸ initial feed; • labeled feed.

(Figure 3.8). SPOM $\delta^{15}N$ values for the tank were similar to tanks described above (day 22 $\delta^{15}N = 91.5 \pm 3.3‰$).

The addition of stickwater to the feeds produced results dependent on the concentration added. Shrimp receiving the lower concentration showed the smallest uptake of all of the outdoor trials. Muscle and carapace $\delta^{15}N$ values averaged 43.7 ± 1.1‰ and 47.5 ± 0.6‰ on day 22 (feed label = 104.8‰; Figure 3.9). The day 22 $\delta^{15}N$ values of SPOM in these tanks were also significantly lower than any of the other outdoor trials (68.3 ± 5.3‰; one-way ANOVA combined with Tukey procedure; $p = 0.01$). In contrast, the shrimp showed the highest muscle and carapace $\delta^{15}N$ values when stickwater was added as 2.5% of the weight of the feed (average $\delta^{15}N$ values of 57.2 ± 0.2‰ and 65.4 ± 0.5‰ for muscle and carapace, respectively; feed label = 115.0‰; Figure 3.10). The final SPOM $\delta^{15}N$ value of 89.1 ± 2.2‰ was similar to those in the first two trials.

As with the indoor trials, carbon values for muscle and carapace in each of the treatments changed little over time (average values of -19.8‰ to -20.2‰ for muscle and –19.4‰ to –18.9‰ for carapace). However, the average $\delta^{13}C$ values for SPOM increased over the course of the study from –28.7 ± 0.6‰ to –20.7 ± 1.1‰. This trend likely indicated different algal species succession.

Percent Label in Muscle

Indoor

The percent label values for both the indoor and outdoor trials are shown in Table 3.3. Day 22 values for indoor trials varied from 7.7 ± 2.1% for 'green' feed with SLP to 11.7 ± 0.1% for shrimp fed the standard fishmeal feed. However, one-way ANOVA analysis indicated no difference among day 22 values for all treatments.

Outdoor

Shrimp in the outdoor trials assimilated the label at a higher rate than shrimp in the indoor trials due to their more rapid growth as well as the potential for nitrogen recycling within the outdoor tanks. The values for the outdoor trials ranged from 27.4 ± 0.5% for shrimp receiving 'green' feed without an attractant/stimulant to 34.6 ± 1.0% in shrimp fed 2.5% stickwater amended 'green' rations. Statistically, however, percent label was again not significantly different between treatments.

DISCUSSION

Effective chemoattractants and feeding stimulants would be extremely beneficial in commercial aquaculture systems where the cost of formulated feeds can be high (Lee and Meyers, 1996; Lee and Meyers, 1997). In the outdoor systems employed here, the reliance of the shrimp on natural production within the tanks is significant and sustained as shown by improved growth rates in the presence of tank primary and secondary

Table 3.3: Percent label assimilation by shrimp for day 22 for indoor and outdoor attractant/stimulant trials.

Treatment	n	Indoor	Outdoor
Green feed + SLP	2	7.73 (2.08)	29.2 (3.6)
Green minus SLP	2	8.90 (0.52)	27.4 (0.5)
Green + 1% Stickwater	2	9.44 (0.90)	30.8 (3.1)
Green + 2.5% Stickwater	2	7.81 (0.94)	34.6 (1.0)
Control feed (fish meal)	2	11.7 (0.1)	

Numbers in ( ) represent population standard deviation.
n = number of tanks in trial.

producers (Anderson et al., 1987; Moss and Pruder, 1995; Moss et al., 1992; Nunes et al., 1997; Parker et al., 1989; Schroeder et al., 1984; Schroeder, 1983a) (see also Chapter 4). In this system, attractants and feeding stimulants would be particularly important for feed detection and utilization by the shrimp.

The indoor trials were performed to assess the shrimp response to stickwater amended feed within controlled conditions. However, the results were inconclusive. Feed assimilation and growth rates were slightly higher for shrimp receiving the fishmeal protein feed. However, the differences between 'green' feeds with or without added attractants/stimulants were minimal and insignificant. Highest assimilation and growth rates were found in 'green' tanks receiving 1% stickwater and those receiving feed without an added attractant. The fact that highest percent label uptake was found on feed containing fishmeal is not surprising, as this is a known high quality protein source. Shrimp utilize some plant material in their diet, but the amount declines as they grow (Rothlisberg, 1998). The similar uptake and growth by shrimp receiving feed with and without the standard attractant, as well as with and without stickwater, may have been due to the operating conditions for the indoor tanks that required water turnover of 100% volume/hour. This rapid turnover would limit the time that an attractant was available to the shrimp.

In the outdoor trials with the added influence of natural production, lowest growth and percent label were found for shrimp receiving feed without an added attractant/stimulant. While the differences between outdoor feeding trials were not statistically significant, percent label values and growth rates were highest for shrimp receiving the maximal amount of stickwater. The high percent label values for 2.5% stickwater amended feeds suggested it may be useful in enhancing feed uptake in natural conditions and further experiments are warranted.

Other researchers who have examined performance indicators as part of attractant/stimulant studies found varied results. Some researchers feel that proteins do not make good attractants due to slow leaching (Heinen, 1980), but stickwater contains many soluble components and may be useful as an attractant. Pascual (1980) investigated the response of *Penaeus monodon* to feeds containing squid, mussel, shrimp and fish extracts. She found the highest growth rates on feeds containing mussel extracts, but the best survival with shrimp extracts. Nevertheless, she concluded that mussel extracts would act as the best attractant particularly given the costs of squid extracts. As with this study, she noted high feed utilization on feed with fish extracts. Similar to the findings presented here, Hartati and Briggs (1993) found comparable assimilation rates for *Penaeus monodon* given feeds with different

attractants/stimulants. They concluded that taurine and an amino acid mix were the best attractants based on separately tested behavioral responses.

Most of the work examining the role of a substance as an attractant or feeding stimulant for shrimp has been done using behavioral cues (Carr and Derby, 1986; Costero and Meyers, 1993; Zimmer-Faust, 1987). By comprehensively surveying the response of shrimp to a given food with different additives, a substance can be characterized as attractant, stimulant, or enhancer (Lee and Meyers, 1996). This study did not attempt to catalog the behavioral response of the shrimp to the amended feeds, and it is difficult to compare the results obtained here to other works. While stable isotope enrichment experiments give insight into feed utilization, it is suggested that concurrent behavioral trials, such as those described by Costero and Meyers (1993), would be useful in fully understanding the role of a potential attractant. Furthermore, it would be important to chemically characterize stickwater in comparison to the standard attractant, squid liver powder. Different 'batches' of stickwater (i.e. different fish species, catches from various times of the year representing different fitness levels) could have vastly different characteristics, making it more or less attractive to the shrimp (Gaudé, 1994).

CONCLUSIONS

Indoor and outdoor trials to test the usefulness of stickwater as a feeding stimulant indicate a similar response by the shrimp to feed containing stickwater and the standard attractant, squid liver powder, and to feed containing no attractant at all. More experiments are necessary to fully examine the role of stickwater as a feeding stimulant. The percent label incorporation results from the outdoor treatments showed increasing label incorporation at the higher stickwater concentration suggesting that dose/response information may be useful. Future research should investigate the quality of the stickwater produced from differing species of fish at different times of the year as season and growth cycle can affect its composition. Additionally, it is recommended that behavioral trials complement stable isotopic enrichment for a more complete understanding of the efficacy of stickwater as an attractant for shrimp.

CHAPTER 4 - THE ROLE OF TANK NATURAL PRODUCTIVITY IN SHRIMP GROWTH

INTRODUCTION

Determining the nutritional completeness of formulated feeds in shrimp aquaculture is complicated by the enhanced shrimp growth in the presence of natural pond assemblages (Anderson et al., 1987; Leber and Pruder, 1988; Moss and Pruder, 1995; Moss et al., 1992; Nunes et al., 1997; Parker et al., 1989; Rubright et al., 1981; Schroeder et al., 1984; Schroeder, 1983a; Tacon and Akiyama, 1997). Studies of the natural diet of wild species of shrimp indicate that they utilize benthic and pelagic algae in addition to other food sources (Gleason, 1986; Robertson, 1988; Stoner and Zimmerman, 1988). Thus, it is not surprising that previous studies have indicated that up to 86% of shrimp growth carbon is from natural pond biota in semi-intensively reared pond-raised shrimp (Anderson et al., 1987; Nunes et al., 1997; Parker et al., 1989). Nevertheless, shrimp utilization of pond biota varies widely. Schroeder and colleagues (Schroeder et al., 1984; Schroeder, 1983a) found that while pond biota contributed up to 50% of growth carbon for *Macrobrachium rosenbergii*, penaeid species were selectively utilizing feed components with little reliance on pond organic matter (Schroeder, 1983b). While it is unclear as to why the shrimp do better in the

presence of pond biota, it is evident that the relationship between shrimp and algal and bacterial populations needs to be investigated further to determine the specific components that shrimp derive from pond biota. With this information, it may be possible to supply key nutrients via the feed or to ensure enhanced natural production to stimulate shrimp production.

Bacteria, in addition to benthic and pelagic algae, may contribute to shrimp growth. Early studies on shrimp nutrition suggested that bacteria may play a significant role in shrimp growth (Moriarty, 1976; Moriarty, 1977), but more recent studies have discounted the findings (Moss et al., 1992; Moss and Pruder, 1995). However, bacterial processing of feed pellets may indirectly provide nutrients for primary productivity, which would then be consumed by the shrimp. Moriarty (1986) found that in penaeid culture ponds most of the added feed was supporting bacterial growth rather than shrimp growth. The support of the heterotrophic community in this manner led Schroeder (1983b) to suggest that the aquaculture system is in effect a rumen, in which the feeds are converted into algal, bacterial and protozoan biomass, which in turn supports the target species.

Several studies have used natural abundance stable isotope analysis to examine the nutritional importance of formulated feeds and natural pond populations to shrimp

growth (Anderson et al., 1987; Focken and Becker, 1998; Nunes et al., 1997; Parker et al., 1989; Schroeder, 1983a; Schroeder, 1983b). However, there are only a few studies that have used stable isotopic enrichments to obtain information on shrimp nutritional needs (Preston et al., 1996). The primary benefit to using stable isotope enrichments in nutritional studies is the clear isotopic identification of source materials in situations where the subject utilizes more than one food source. In this study, nutrient flows from tank natural populations to intensive cultures of Pacific white shrimp, *Litopenaeus vannamei*, were traced via direct additions ^{15}N and ^{13}C enriched compounds to tank water. The objective of this study was to obtain estimates of the direct or indirect contributions of natural algal and bacterial production to shrimp growth within zero-water exchange culture tanks.

MATERIALS AND METHODS

Study Location and Rearing Conditions

Experiments were carried out in tandem with the labeled feed experiments at the Oceanic Institute, Waimanalo, Hawaii (OI: see Table 1.1). Therefore, the shrimp rearing and sampling schedule were the same as those discussed previously (Chapters 2 and 3). Briefly, outdoor mesocosm laboratories (1200 L for OML 98-1, 1300 L for all others) were stocked with juvenile *Litopenaeus vannamei* ranging in average size from 0.8 g to

3.4 g over four experimental periods. Because of the known importance of natural productivity, tanks were seeded with water from shrimp nursery tanks approximately five days prior to the addition of the shrimp to allow primary productivity to become established. Diatoms usually dominated the OML tank communities. Temperatures over the four experiments ranged from 19.8°C to 30.5°C; dissolved oxygen was 3.8 to 7.4 mg/L; and pH was 6.6 to 8.8 (Table 4.1). The Oceanic Institute staff monitored water quality weekly and calculated shrimp wet weights, feed conversion ratios (FCR), specific growth rates (SGR), and survival for each of the tanks.

Feed Rations and Isotope Additions

In each of the trials described within, the shrimp were fed reference feed rations formulated at OI and described in Chapters 2 and 3. All feed ingredients are listed in Appendix D. In the first three experiments, ^{15}N-ammonium chloride (99% ^{15}N atom enriched) was added in solution to three tanks at 0.1 g/tank (1200 L for OML 98-1, 1300 L for all others) for direct labeling of tank algae. For OML 01-1, 0.83 g was added to each tank. Similarly, ^{13}C-mannitol ($C_6H_{14}O_6$, 99% ^{13}C atom enriched) was added to tanks for OML 99-3 at 0.167 g/tank for direct labeling of tank bacterial populations. These pulse additions of labeled ammonium to tanks occurred on 23 March 1998, 7 October 1998, 2 August 1999, and 11 January 2001 for OML 98-1, 98-5, 99-3, and

Table 4.1: Ranges of tank temperature, pH, and dissolved oxygen for each of the trials.

OML	Temperature (°C)		pH		DO (mg/L)	
	min	max	min	max	min	max
98-1	20.1	26.7	7.8	8.8	3.8	7.4
98-5	22.8	30.5	6.6	8.5	5.1	7.1
99-3	19.8	29.2	7.2	8.6	3.8	7.7
01-1	23.2	29.8	7.8	8.2	6.4	7.9

01-1. Labeled mannitol was added as a single dose to OML 99-3 tanks on 2 August 1999.

Uncovered versus covered tanks

Green Tank Covers

To assess whether increased shrimp growth in the presence of natural tank populations was due to direct consumption by the shrimp or the production of a growth enhancing micronutrient by algal populations, each of the treatments in OML 99-3 was performed under green covered and non-covered conditions. For covered conditions, the OML tanks were overlaid with transparent green plastic film in an attempt to limit algal production. The goal was to inhibit the algae but retain the natural light/dark cycle for the shrimp. As with uncovered tanks from the same trial, $^{15}NH_4Cl$ and ^{13}C-mannitol were added at 0.1 g/tank on 2 August 1999.

Black Tank Covers

Bacterial utilization of NH_4 was tested via a $^{15}NH_4$ addition performed under black plastic covered (algal production inhibited) conditions in OML 01-1. Black covered tanks received the same amount of label (0.83 g of $^{15}NH_4Cl$) as uncovered tanks on 11 January 2001. Light levels under the black covers were reduced to values of 0.02 x 10^{14} quanta cm^{-2} sec^{-1} as compared to ambient levels of 1.45 x 10^{17} quanta cm^{-2} sec^{-1}

(measured with a Biospherical Instruments QSL-100 4 pi light meter). Any uptake and transfer of the label in the dark tanks to the shrimp would be assumed to be via bacterial utilization.

Sampling, Preparation, and Mass Spectrometry

Isotopic samples were taken on the same schedule as the trials involving labeled feed (Chapters 2 and 3). As noted with those trials, all treatments were assigned randomly to three tanks each. Two shrimp were sampled from each of three tanks biweekly for the duration of the experiment. During OML 99-3 and 01-1, shrimp from two of the three tanks were processed for isotopic ratios. Day 1 designates the time at which the label was added to the tanks. On each sampling date, between 10 and 100 ml of tank water was filtered through GF/C filters to collect samples of suspended particulate organic matter (SPOM).

Shrimp were boiled and muscle and carapace tissues separated prior to isotopic analysis in OML 98-1. In the remaining experiments, shrimp muscle was dried, either in a drying oven at 80°C for 48 hours or by freeze-drying overnight. Carapace samples were rinsed in deionized water, fumed in an HCl environment overnight to remove carbonates, and then dried in an oven at 80°C for 24 hours. Filters with SPOM were treated similarly to carapace samples, with acidification and drying.

Samples of shrimp muscle tissue and formulated feeds were ground to a homogenous powder prior to mass spectrometer analysis. Carapace was subsampled and filters were analyzed whole or cut in half depending on the concentration of material. Isotope ratios were obtained on either a Europa 20/20 continuous flow isotope ratio mass spectrometer or a Finnigan Delta Plus mass spectrometer.

Calculation of the Contribution of Natural Production to Shrimp Growth

The relative contribution of natural tank populations versus formulated feed to shrimp growth was determined using equations from Anderson et al. (1987) and Parker et al. (1991). These are as follows:

$$\frac{W_p}{W_f} = \frac{\delta_f - \delta_g}{\delta_g - \delta_p}$$

where W_p is shrimp weight gained from pond (in this case, "tank") production, W_f is weight gain from formulated feed and δ_p, δ_f, and δ_g are isotopic ratios for the pond biota, feed, and growth respectively. The δ_g term is obtained from the equation:

$$\delta_g = (W_t\delta_t - W_i\delta_i)/W_g$$

where i and t represent the initial value and that at time t and W_g is the weight gained (see Appendix E for a sample calculation).

Statistics

All growth and efficiency measures as well as the percent contribution of natural production and percent label reflected in muscle tissue were compared using one-way ANOVA with the Tukey multiple comparisons procedure to test for differences between individual means. For ANOVA, individual shrimp values were averaged for a mean tank value before using these tank values as the repeated measure. FCR values were log transformed prior to ANOVA. Nutrients for each of the experimental tanks were compared to their respective control tanks using a general linear model (GLM) in which both day and treatment were used to model nutrient concentrations (Minitab version 8.21 for Macintosh). Carapace and muscle $\delta^{15}N$ and $\delta^{13}C$ values from the same animals were compared using a paired Student's t-test, in which $p < 0.05$ was considered significant. The increases in muscle, carapace, and SPOM $\delta^{15}N$ and $\delta^{13}C$ values for a given treatment were compared to their respective controls via an analysis of slopes as described in Chapter 2. By only comparing between treatments, the assumption of independence is satisfied. Due to dilution of the one time label addition, slope comparisons were only done using the initial few days, until the tissue reached maximum isotopic ratios. Mean values were reported with population standard deviation (i.e., mean ± standard deviation).

RESULTS

Growth and Efficiency Measures

The growth and efficiency measures for shrimp receiving pulse additions of isotopic label directly to tank water are given in Table 4.2. Values from each of the respective control tanks (discussed more completely in Chapter 2 for OML 98-1, 98-5, and 99-3 uncovered) are shown for comparative purposes. Statistically, each of the isotope addition tanks had similar efficiency measures as their respective control tanks (one-way ANOVA combined with Tukey procedure; $p > 0.05$). The shrimp grown in the covered tanks of a given experiment had growth rates similar to those in uncovered tanks from the same experiment. Growth rates varied considerably from 0.5 g/wk for 'black' covered OML 01-1 tanks to 1.9 g/wk for 'green' covered tanks in OML 99-3. Shrimp in trials OML 98-5 and 99-3 had similar growth rates. Growth rates in all treatments from OML 98-5 and 99-3 were significantly higher than those from OML 98-1 and OML 01-1 (one-way ANOVA combined with Tukey procedure; $p < 0.001$). SGR (1.7 to 3.9%/day for OML 01-1 and OML 99-3 respectively) showed a similar trend in values as growth and similar statistical results as well (one-way ANOVA combined with Tukey procedure; $p < 0.001$).

Survival within each of the experiments was highly variable, ranging from 7.5 ± 4.8% to 94.3 ± 4.5% for isotope addition tanks. Survival in OML 98-1 was significantly

Table 4.2: Average shrimp growth and efficiency measures for pulse additions of $^{15}NH_4Cl$ experiments.

OML	Treatment	n	Initial Wt (g)	Final Wt (g)	Weight Gain (%)	Growth (g/wk)	FCR (g feed/g wet wt shrimp)	SGR (%/day)	Survival (%)
98-1	Control	4	3.36 (0.13)	8.70 (0.73)	159 (18)	0.76 (0.10)	3.35 (0.31)	1.94 (0.14)	93.6 (4.0)
	$^{15}NH_4Cl$	3	3.35 (0.25)	8.60 (0.43)	157 (6)	0.75 (0.03)	3.49 (0.15)	1.92 (0.05)	90.4 (0.9)
98-5	Control	2	1.12 (0.01)	14.1	1170	1.08	14.4	3.02	16.7 (23.6)
	$^{15}NH_4Cl$	3	1.09 (0.02)	17.5 (0.8)	1500 (98)	1.37 (0.07)	10.9 (4.8)	3.30 (0.07)	37.8 (12.6)
99-3	Control	2	1.91 (0.04)	15.8 (1.0)	731 (69)	1.74 (0.13)	2.85 (0.25)	3.78 (0.15)	62.3 (1.0)
	$^{15}NH_4Cl$	2	1.89 (0.00)	15.6 (0.5)	723 (27)	1.71 (0.06)	3.94 (2.68)	3.76 (0.06)	60.3 (42.6)
	^{13}C-mannitol	2	2.01 (0.03)	12.6 (4.9)	525 (237)	1.32 (0.61)	21.9 (26.1)	3.21 (0.69)	28.8 (29.1)
99-3	Control	2	1.97 (0.03)	17.0 (0.9)	762 (30)	1.88 (0.10)	4.53 (3.47)	3.85 (0.06)	50.7 (36.8)
Green	$^{15}NH_4Cl$	2	1.94 (0.07)	15.5 (1.9)	700 (127)	1.69 (0.25)	31.6 (23.8)	3.70 (0.28)	7.53 (4.84)
Covers	^{13}C-mannitol	2	1.95 (0.07)	17.2 (0.1)	782 (26)	1.91 (0.00)	2.52 (0.82)	3.89 (0.05)	65.8 (21.3)
01-1	Control	2	0.85 (0.00)	3.31 (0.24)	291 (26)	0.62 (0.06)	1.27 (0.17)	1.95 (0.09)	95.6 (4.5)
	$^{15}NH_4Cl$	2	0.79 (0.01)	3.56 (0.30)	352 (46)	0.70 (0.08)	2.17	2.15 (0.14)	37.3 (31.5)
01-1 Black Covers	$^{15}NH_4Cl$	2	0.83 (0.04)	2.71 (0.09)	229 (27)	0.47 (0.03)	1.67 (0.19)	1.70 (0.11)	94.3 (4.5)

Numbers in ( ) represent population standard deviation.
n = number of tanks in trial.
n = 1 tank for OML 98-5 control final weight, weight gain, growth, FCR, SGR and OML 01-1 $^{15}NH_4Cl$ FCR.

higher than that in OML 98-5 and OML 99-3 (one-way ANOVA combined with Tukey procedure, $p = 0.002$). Survival in OML 01-1 was mixed, with low survival in uncovered NH_4 tanks ($37.3 \pm 31.5\%$) and high survival in black covered NH_4^+ tanks ($94.3 \pm 4.5\%$). However, the large variations in survival values among OML 01-1 tanks resulted in a lack of significant differences among trials. FCR values were also quite variable, with tanks ranging from 1.3 g feed/g wet weight of shrimp to 31.6. As with survival, there were no significant differences among trials.

Nutrient Profiles

GLM analysis for each of the trials indicated that the nutrient concentrations in the NH_4^+ addition tanks were similar to those in the control tanks (Figures 4.1-4.5). Actual NH_4^+ additions were less than 1% of the amount present in the tanks, and rapid uptake quickly reduced concentrations to background levels. Increases in $NO_2^- + NO_3^-$ concentrations concurrent with declines in TAN suggested that nitrification was occurring in these tanks. There was an increase in nutrients throughout the experiment in the black tanks from OML 01-1. The concentrations of both TAN and $NO_2^- + NO_3^-$ in the black tanks resulted in a significant effect for treatment in GLM analysis ($p < 0.001$). Average TAN values ranged from 0.06 mg/l for uncovered tanks in OML 01-1 to 3.26 mg/l for OML 98-1. The average $NO_2^- + NO_3^-$ ranged from 0.15 mg/l for OML

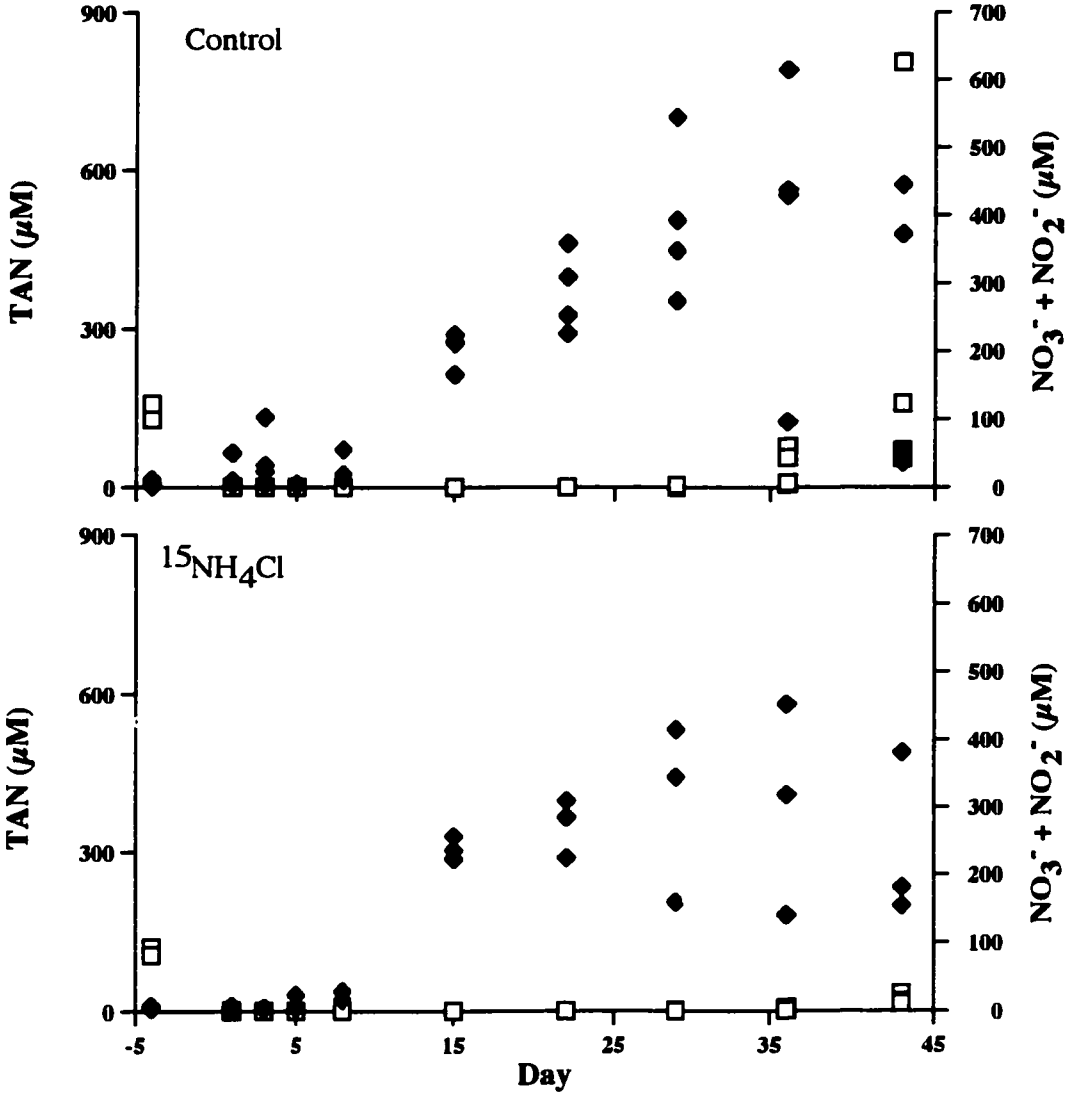

Figure 4.1: Nutrient profiles for OML 98-1. □ $NO_3^- + NO_2^-$; ◆ TAN.

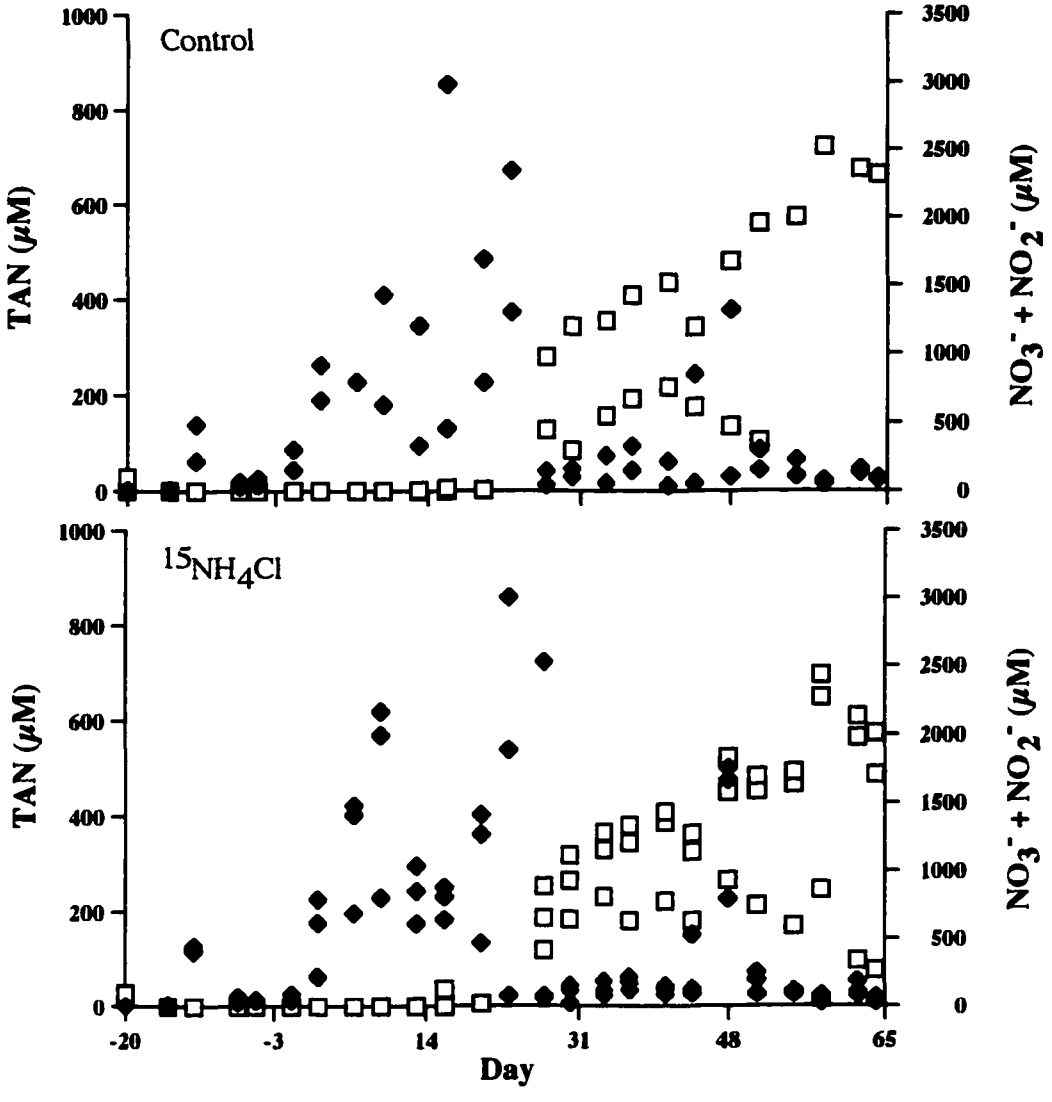

Figure 4.2: Nutrient profiles for OML 98-5. □ $NO_3^- + NO_2^-$; ◆ TAN.

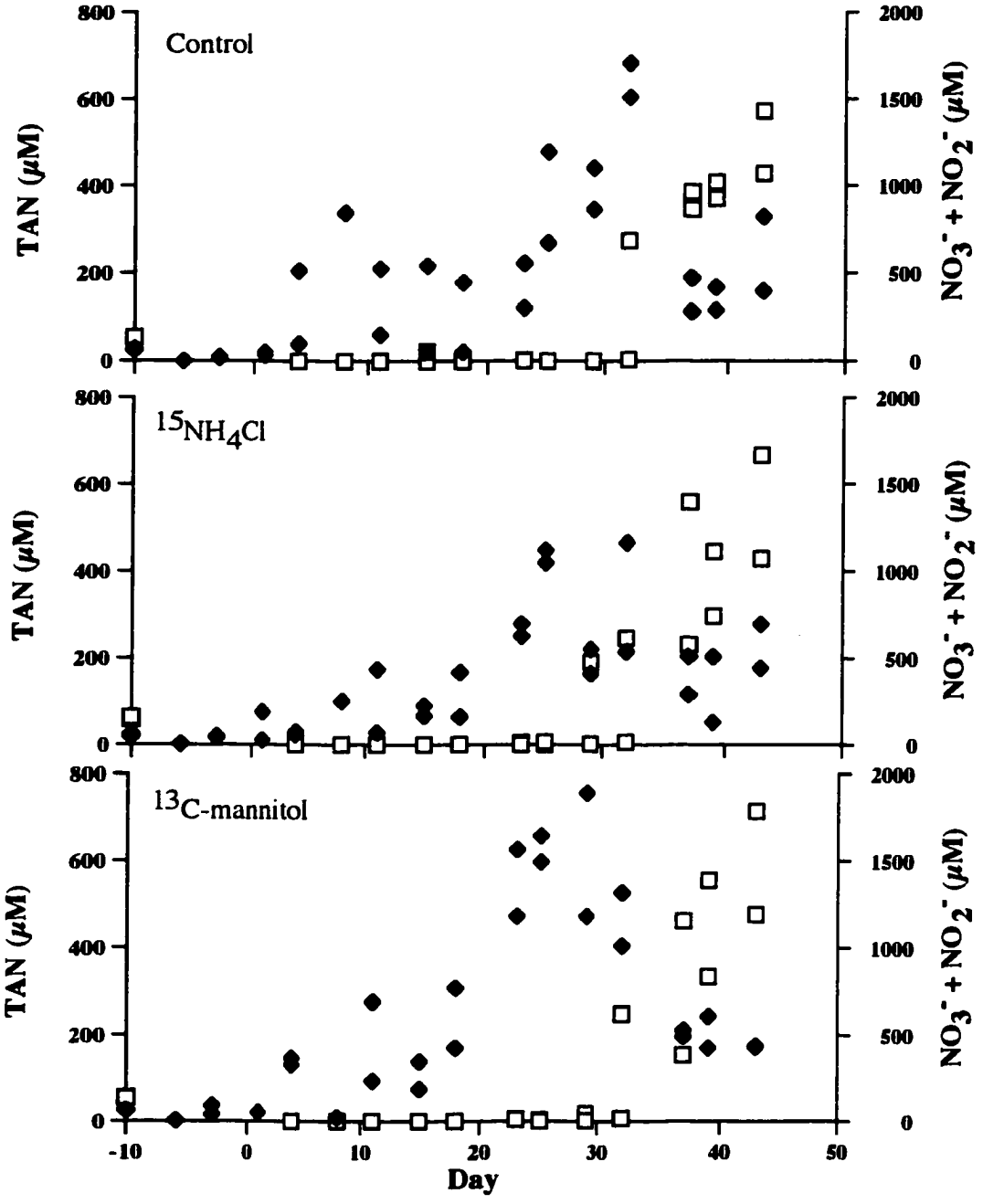

Figure 4.3: Nutrient profiles for OML 99-3, uncovered tanks. □ $NO_3^- + NO_2^-$; ◆ TAN.

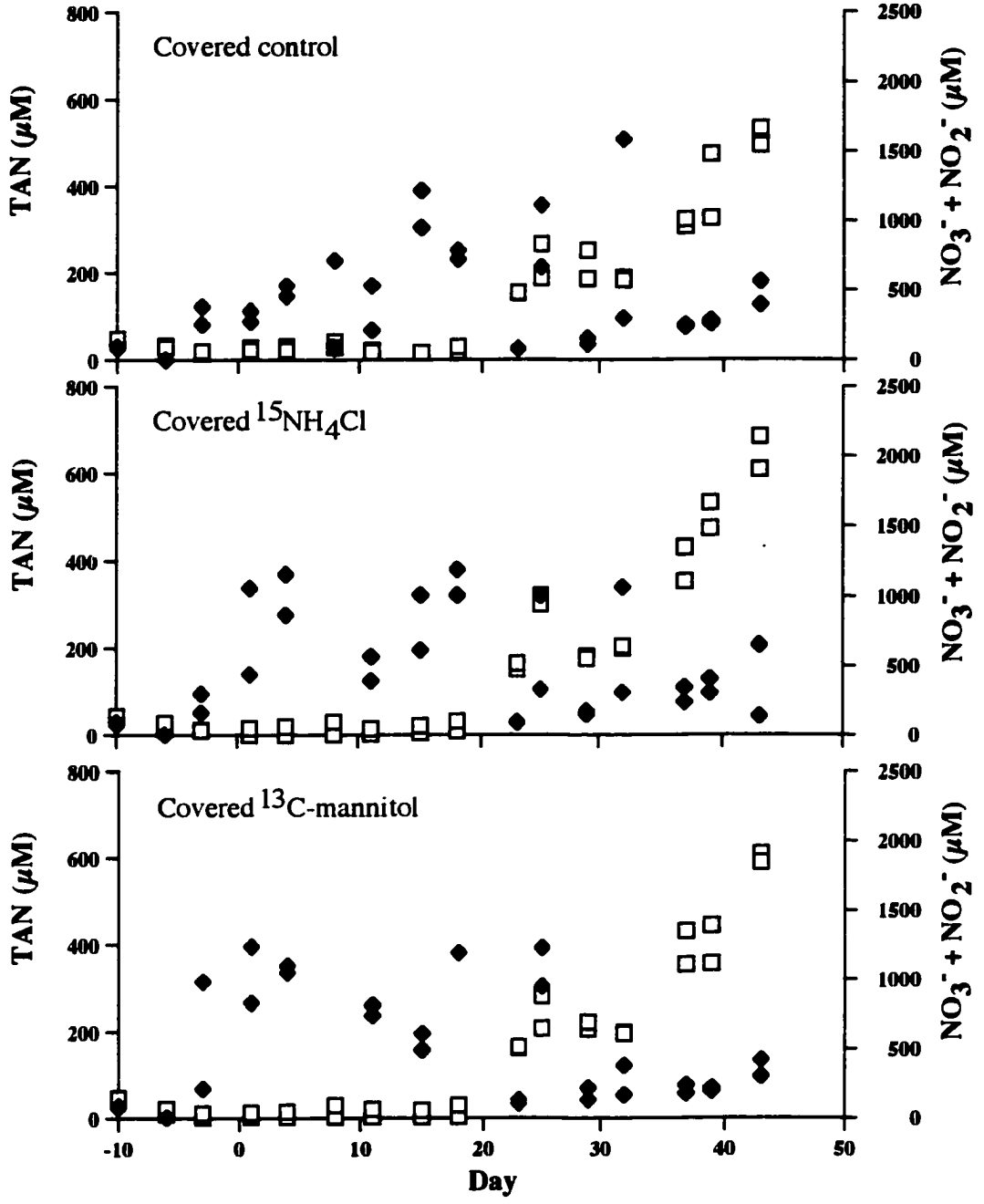

Figure 4.4: Nutrient profiles for OML 99-3, covered tanks. □ $NO_3^- + NO_2^-$; ◆ TAN.

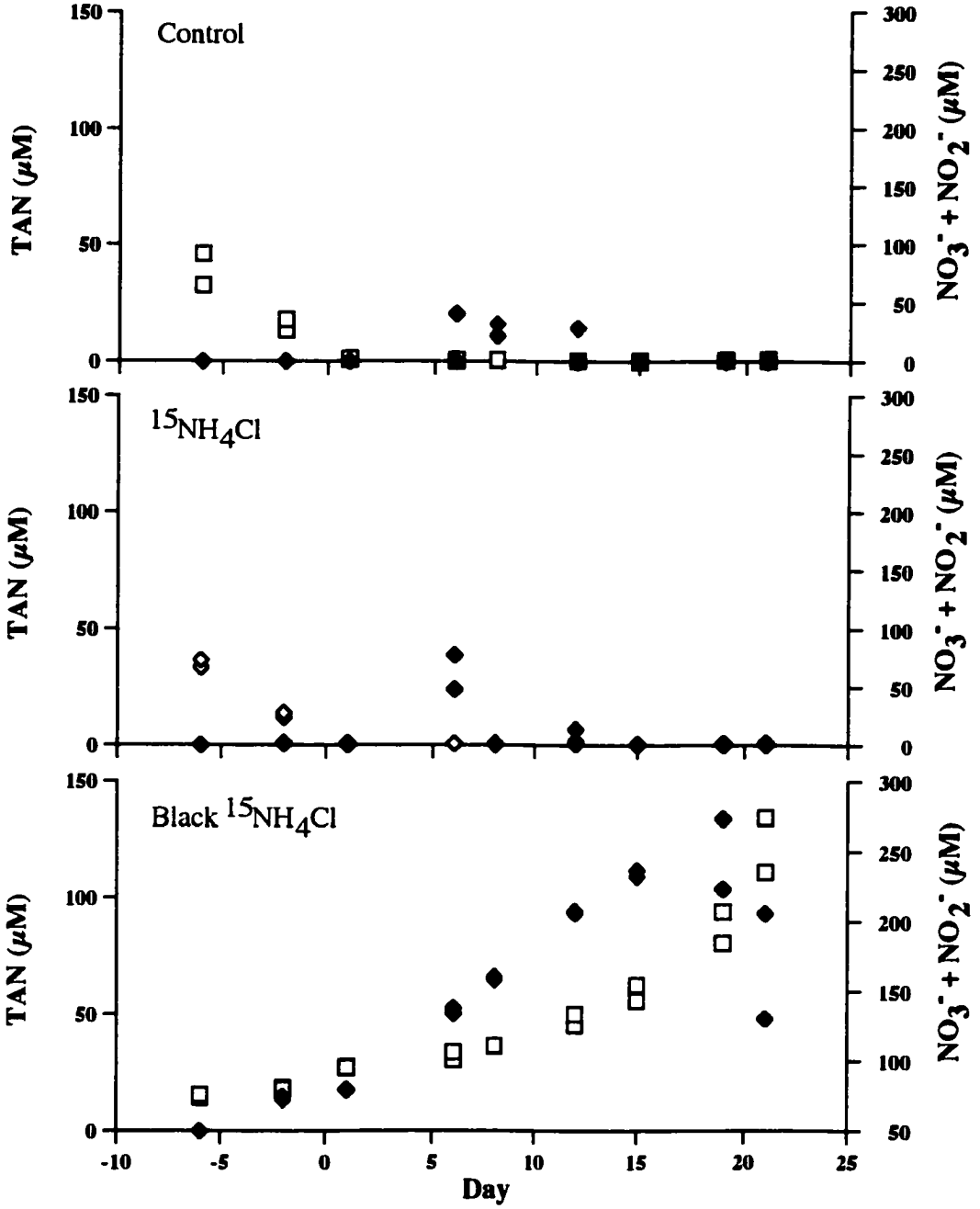

Figure 4.5: Nutrient profiles for OML 01-1. □ $NO_3^- + NO_2^-$; ◆ TAN.

98-1 to 7.0 mg/l for OML 99-3 covered tanks.

Isotopic Trends

$^{15}NH_4Cl$ Pulse Additions

OML 98-1

SPOM showed an immediate and significant response to the label addition in OML 98-1, reaching a peak $\delta^{15}N$ value of 778.8‰ (average ± 55.4‰ standard deviation) two days after the addition (Figure 4.6). Muscle and carapace $\delta^{15}N$ also increased rapidly after the nutrient was added, reaching maximal average values of 58.9‰ ± 8.3‰ and 97.1‰ ± 10.5‰, respectively, by day 22. These increases were statistically significant when compared to OML 98-1 controls (slope analysis; overall $p < 0.05$, individual comparisons $p < 0.008$). $\delta^{13}C$ values for muscle, carapace, and SPOM were similar to those of the OML 98-1 control tanks (Figure 2.4).

OML 98-5

The addition of $^{15}NH_4Cl$ to tanks in October 1998 produced a more rapid change in shrimp isotope values than was seen in OML 98-1 (Figure 4.7). Average muscle values were 86.0‰ ± 10.4‰ after 12 days. Carapace and tank SPOM showed a similar trend of rapid increase followed by similarly quick dilution of the label by feed derived unlabeled nitrogen. Carapace maximal values reached 178.4 ± 53.7‰ within 5 days of

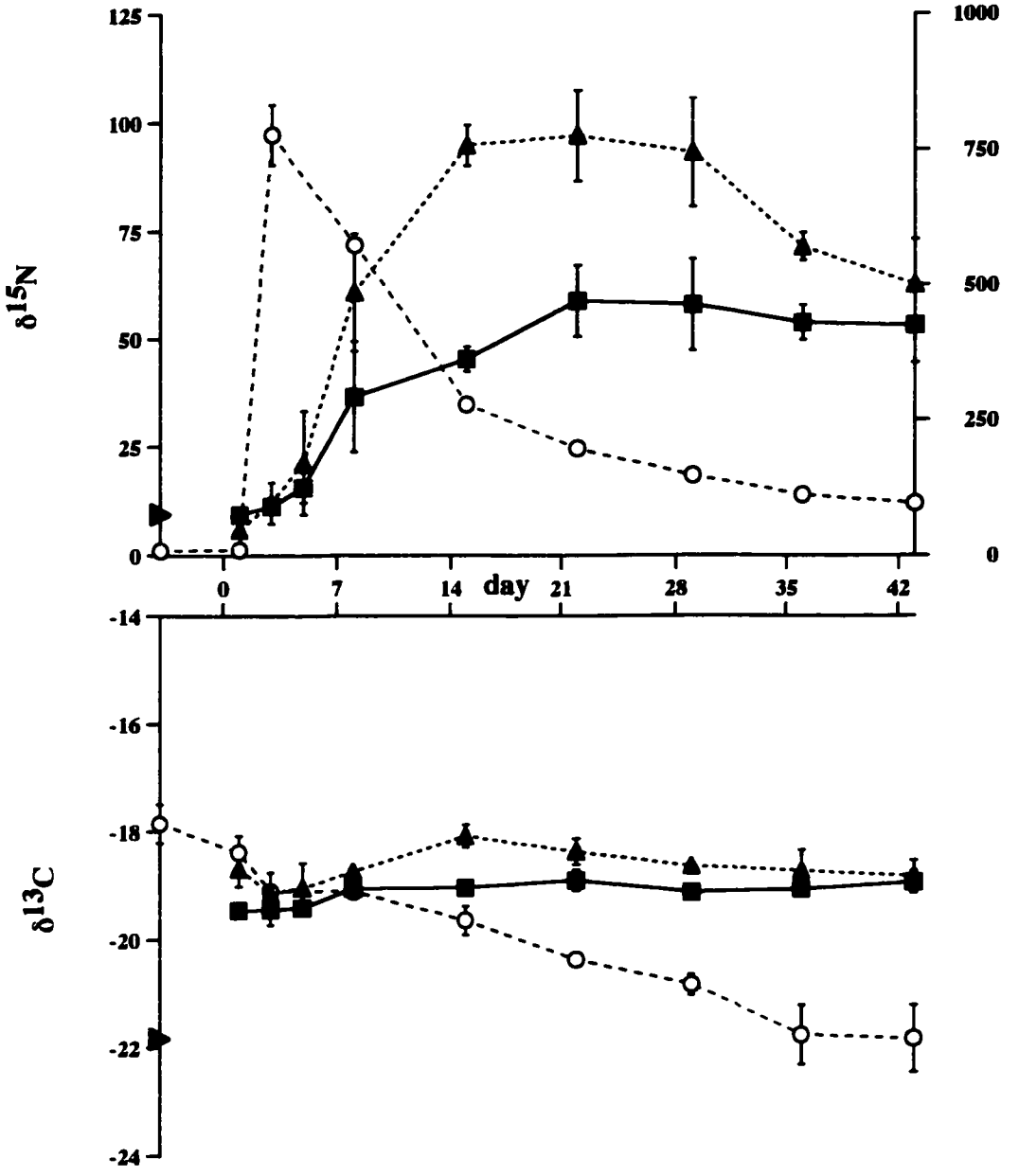

Figure 4.6: $\delta^{15}N$ and $\delta^{13}C$ ratios for OML 98-1 tanks receiving a one time addition of $^{15}NH_4Cl$. N = 3 tanks for muscle, carapace and SPOM each day. SPOM $\delta^{15}N$ values are on secondary y-axis. Error bars represent population standard deviations. ■ muscle; ▲ carapace; O SPOM; ▸ feed.

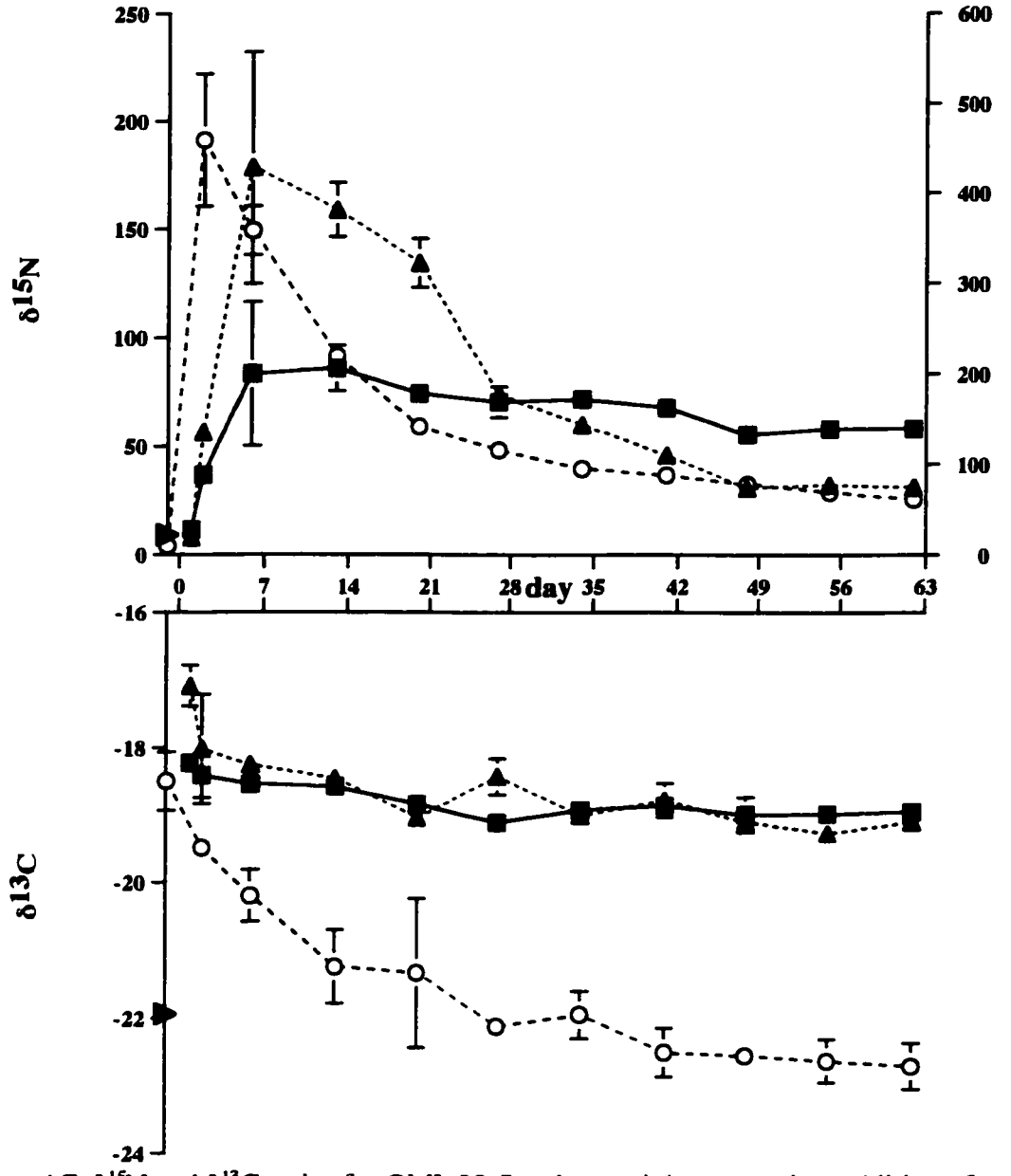

Figure 4.7: $\delta^{15}N$ and $\delta^{13}C$ ratios for OML 98-5 tanks receiving a one time addition of $^{15}NH_4Cl$. N = 3 tanks for muscle, carapace and SPOM each day (except n = 2 for muscle $\delta^{15}N$ and $\delta^{13}C$ on day 6 and muscle and carapace $\delta^{15}N$ and $\delta^{13}C$ values on day 1). SPOM $\delta^{15}N$ values are on secondary y-axis. Error bars represent population standard deviations. ■ muscle; ▲ carapace; O SPOM; ▸ feed.

the addition, and SPOM was 458.8 ± 74.0‰ one day after $^{15}NH_4Cl$ was added. The increases to maximum values for $\delta^{15}N$ of muscle, carapace, and SPOM were significantly different from those of control tanks over the same time period (slope analysis; overall $p < 0.05$, individual comparisons $p < 0.008$). The $\delta^{13}C$ for muscle, carapace, and SPOM track control values (Figure 2.5).

OML 99-3

The response to the addition of $^{15}NH_4Cl$ in OML 99-3 was not as marked as with the previous NH_4^+ additions (Figure 4.8). Carapace and muscle tissue reached maximum $\delta^{15}N$ values 10 days after the pulse addition to the tank water (muscle = 34.9‰ ± 24.1‰ and carapace = 59.9‰ ± 33.7‰). $\delta^{15}N$ values of SPOM reached a maximum $\delta^{15}N$ value of 291.2‰ ± 34.2‰ 3 days after the addition. Statistically, only the increase in SPOM $\delta^{15}N$ for the NH_4^+ addition tanks was significantly higher than its respective control, due to the large number of comparisons associated with the Bonferroni procedure (slope analysis; overall $p < 0.05$, individual comparisons $p < 0.008$). The $\delta^{13}C$ for muscle, carapace, and SPOM track control values (Figure 2.6).

OML 01-1

The response of the uncovered tanks to $^{15}NH_4Cl$ addition in OML 01-1 is shown in Figure 4.9. Because of the large amount of label that was added, standard deviations

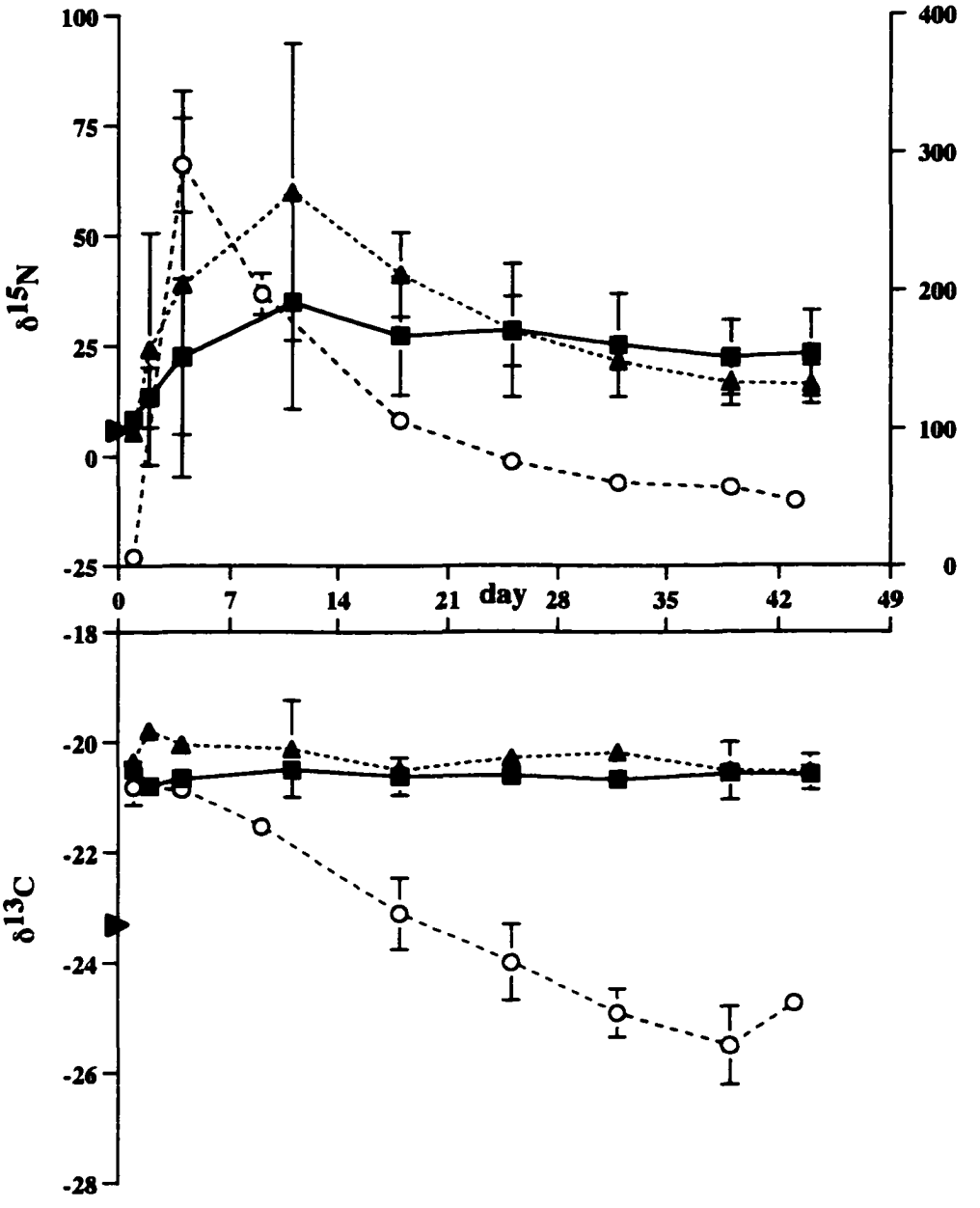

Figure 4.8: $\delta^{15}N$ and $\delta^{13}C$ ratios for uncovered OML 99-3 tanks receiving a one time addition of $^{15}NH_4Cl$. N = 2 tanks for muscle, carapace and SPOM each day. SPOM $\delta^{15}N$ values are on secondary y-axis. Error bars represent population standard deviations. ■ muscle; ▲ carapace; O SPOM; ▸ feed.

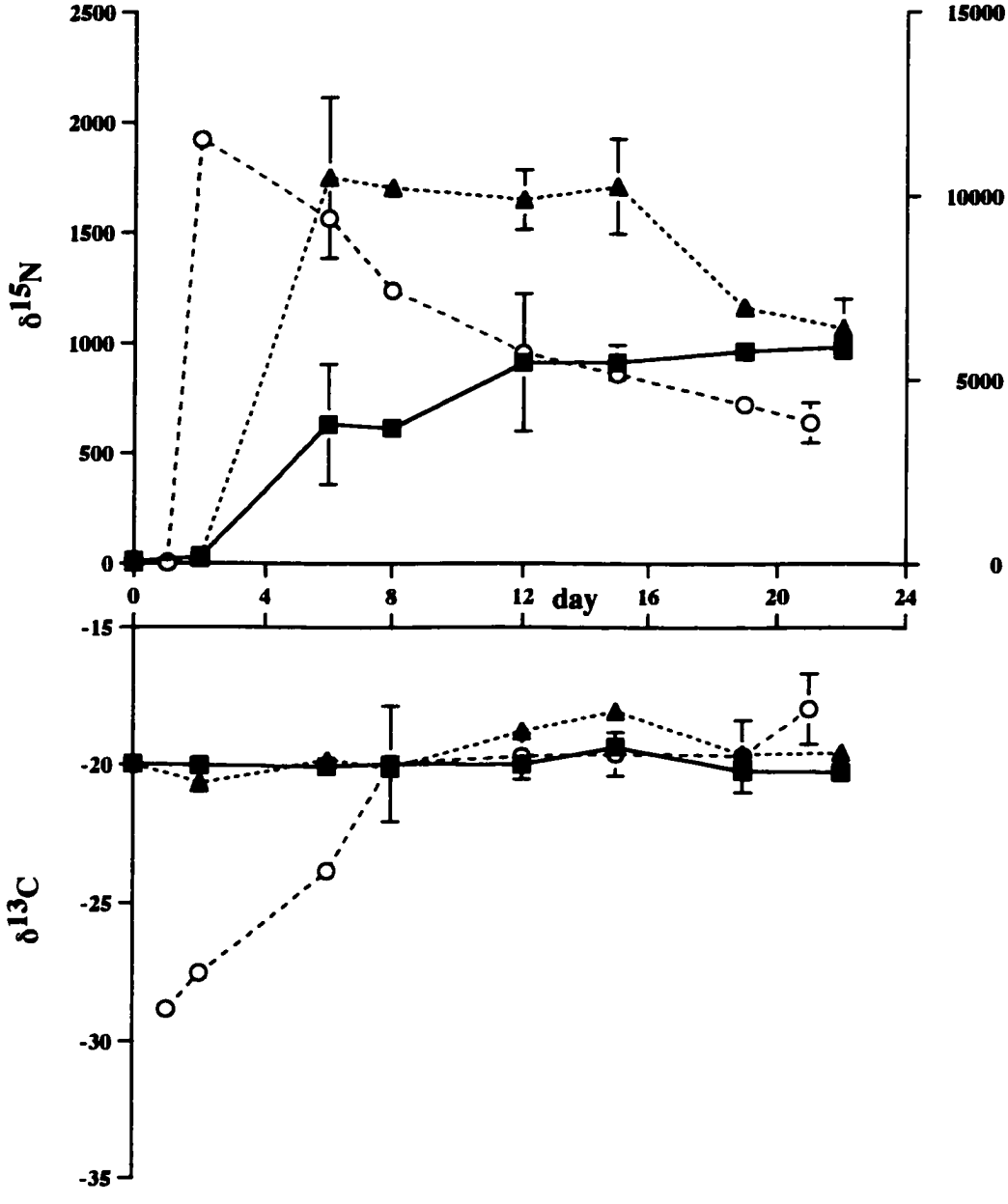

Figure 4.9: $\delta^{15}N$ and $\delta^{13}C$ ratios for uncovered OML 01-1 tanks receiving a one time addition of $^{15}NH_4Cl$. N = 2 tanks for muscle, carapace and SPOM each day. SPOM $\delta^{15}N$ values are on secondary y-axis. Error bars represent population standard deviations. ■ muscle; ▲ carapace; O SPOM; ▸ feed.

are high, but the trends in $\delta^{15}N$ for carapace and SPOM are similar to those of previous experiments. SPOM reached a maximum $\delta^{15}N$ within 1 day of the label addition (11500 ± 307‰) indicating a rapid uptake of NH_4^+, and carapace took only 5 days (1750 ± 366‰). Muscle samples, on the other hand, increased throughout the experiment, reaching 980.3 ± 64.6‰ twenty-one days after the addition. The increases in $\delta^{15}N$ values for carapace and SPOM were statistically greater than the changes found in OML 01-1's control tanks (slope analysis; overall $p < 0.05$, individual comparisons $p < 0.003$; Figure 4.10). The $\delta^{13}C$ values for shrimp muscle, carapace, and SPOM were similar to those of controls. The natural abundance $\delta^{13}C$ of SPOM increased slightly in each of the OML 01-1 treatments, likely due to different species succession of algae over the course of the experiment.

^{13}C-Mannitol Pulse Addition

The response of the shrimp and SPOM to the addition of ^{13}C-mannitol in OML 99-3 is given in Figure 4.11. The daily trends for $\delta^{15}N$ (with no addition of ^{15}N) and $\delta^{13}C$ values of both muscle and carapace were similar to those of control tanks (Figure 2.6). SPOM $\delta^{15}N$ values were also similar to controls. The $\delta^{13}C$ values showed an increase due to the ^{13}C-label addition from –20.7‰ ± 0.02‰ on the day of addition to –15.8‰ ± 1.0‰ by day 4. However, this increase was not significantly different from the trend in

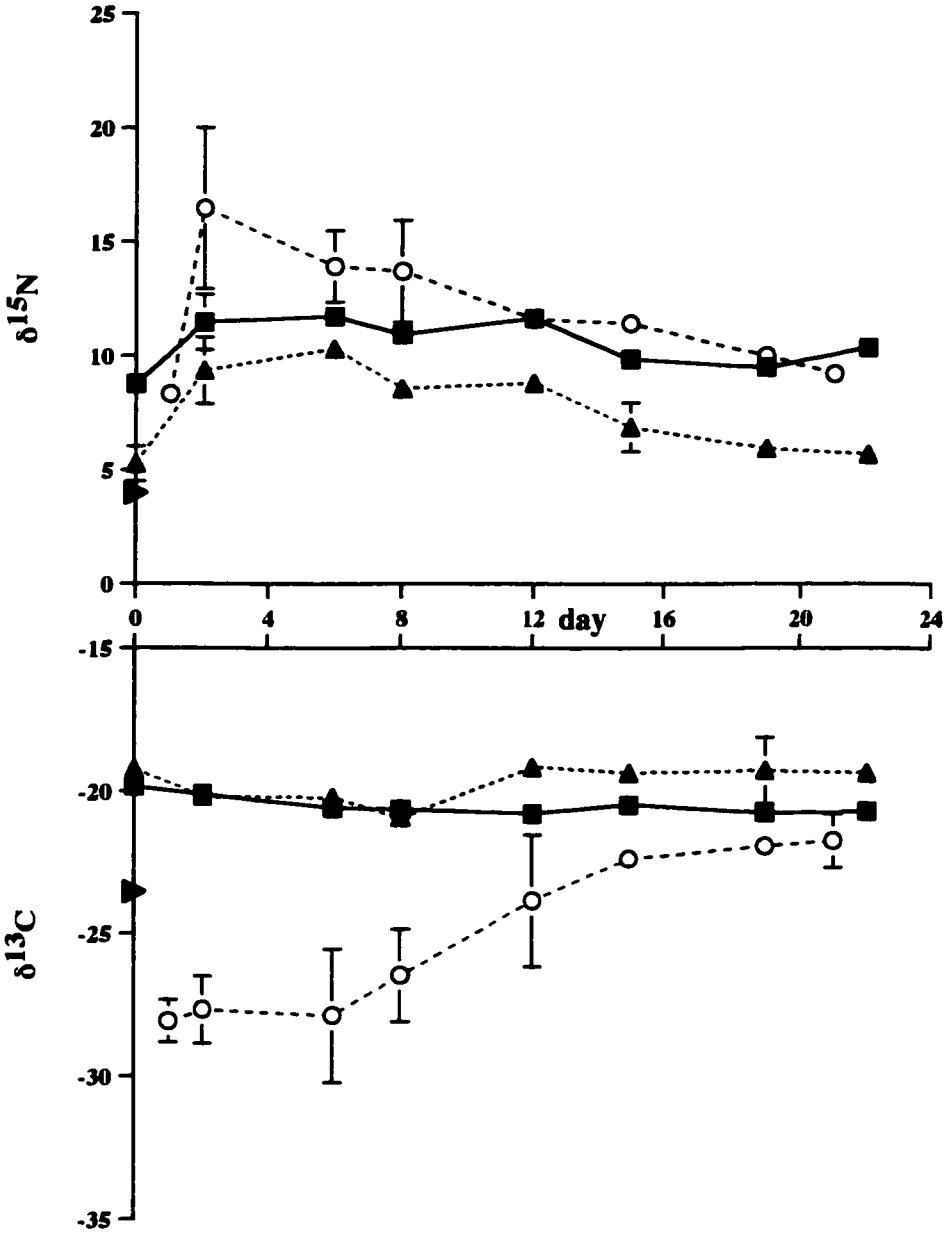

Figure 4.10: $\delta^{15}N$ and $\delta^{13}C$ ratios for control tanks in OML 01-1. N = 2 tanks for muscle, carapace and SPOM each day. Error bars represent population standard deviations. ■ muscle; ▲ carapace; O SPOM; ▸ feed.

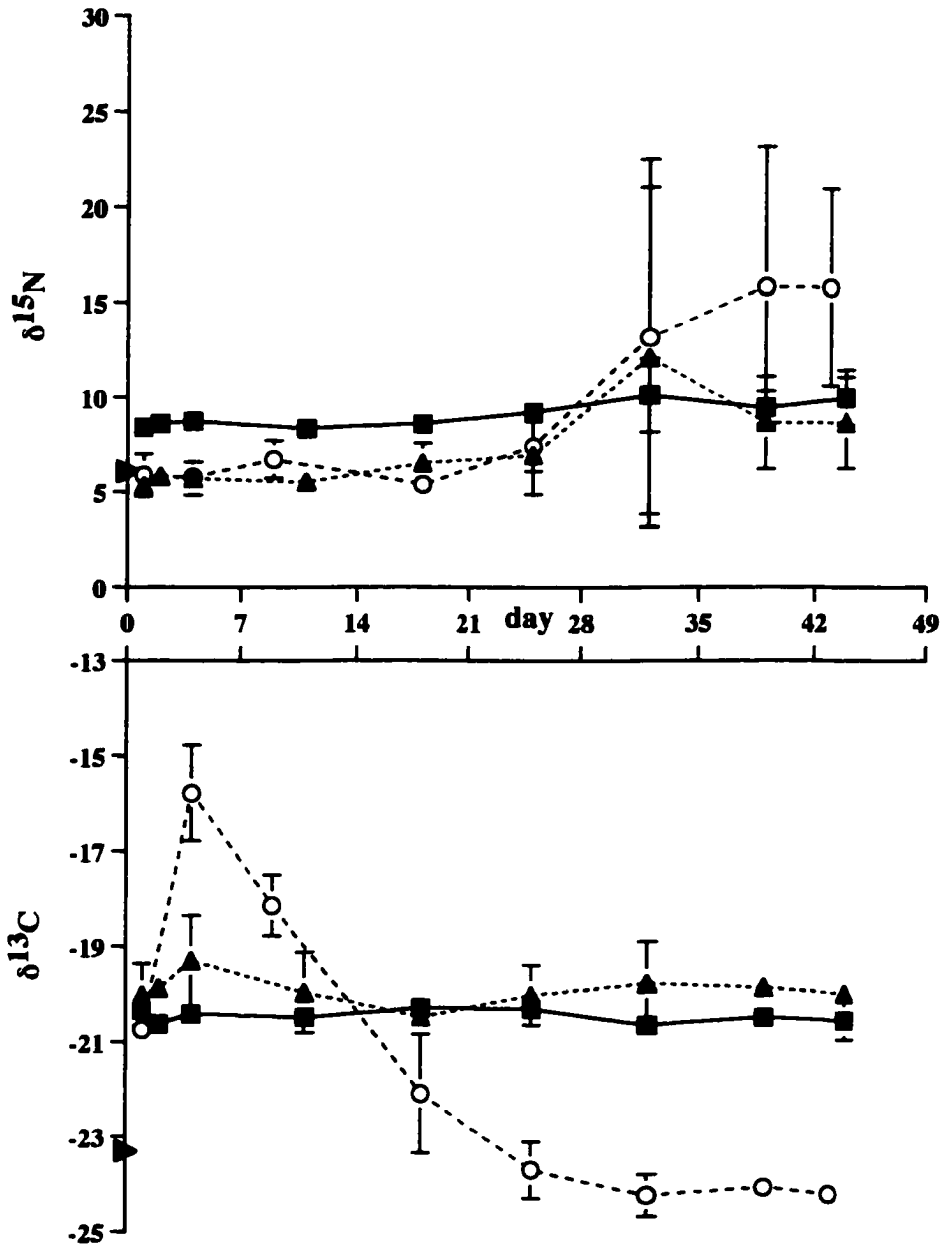

Figure 4.11: $\delta^{15}N$ and $\delta^{13}C$ ratios for uncovered OML 99-3 tanks receiving a one time addition of ^{13}C-mannitol. N = 2 tanks for muscle, carapace and SPOM each day. Error bars represent population standard deviations. ■ muscle; ▲ carapace; O SPOM; ▶ feed.

control $\delta^{13}C$ values over the same period. After day 4, $\delta^{13}C$ values declined to match those found in the control tanks.

Covered Tanks

OML 99-3

The isotope results for shrimp and SPOM in tanks with green plastic film covers are given in Figures 4.12 – 4.14. In covered tanks receiving control feeds, shrimp muscle and carapace values for $\delta^{15}N$ and $\delta^{13}C$ (Figure 4.12) were similar to uncovered control values based on comparison of the slopes of day vs. $\delta^{15}N$ or $\delta^{13}C$ (Figure 2.6). The trends in SPOM $\delta^{15}N$ values were also not significantly different between covered and uncovered tanks. While covered $\delta^{13}C$ values were depleted over the first 18 days when compared to uncovered tanks, values over the length of the experiment were not significantly different.

Green covered tanks receiving the one time addition of $^{15}NH_4Cl$ had more variable isotope ratios, particularly for shrimp carapace and SPOM. As with the uncovered tanks, maximal $\delta^{15}N$ values for muscle (27.2 ± 0.0‰) and carapace (87.6 ± 22.9‰) were found on day 11 and maximal SPOM values (233.2 ± 36.3‰) on day 4. Additionally, the increases in $\delta^{15}N$ for muscle, carapace and SPOM were significantly different from their respective controls (slope analysis; overall $p < 0.05$, individual

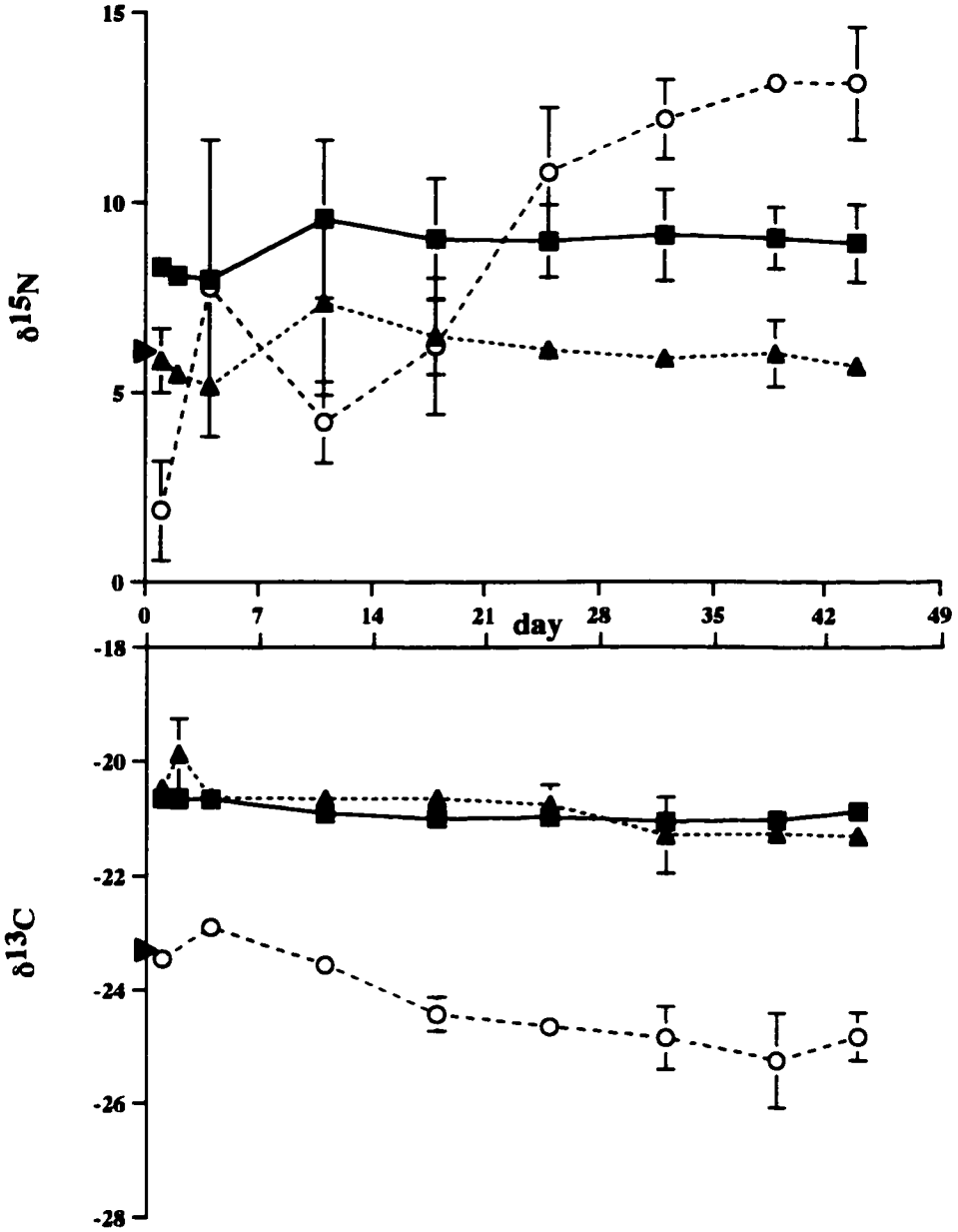

Figure 4.12: $\delta^{15}N$ and $\delta^{13}C$ ratios for green covered control tanks from OML 99-3. N = 2 tanks for muscle, carapace and SPOM each day. Error bars represent population standard deviations. ■ muscle; ▲ carapace; O SPOM; ▸ feed.

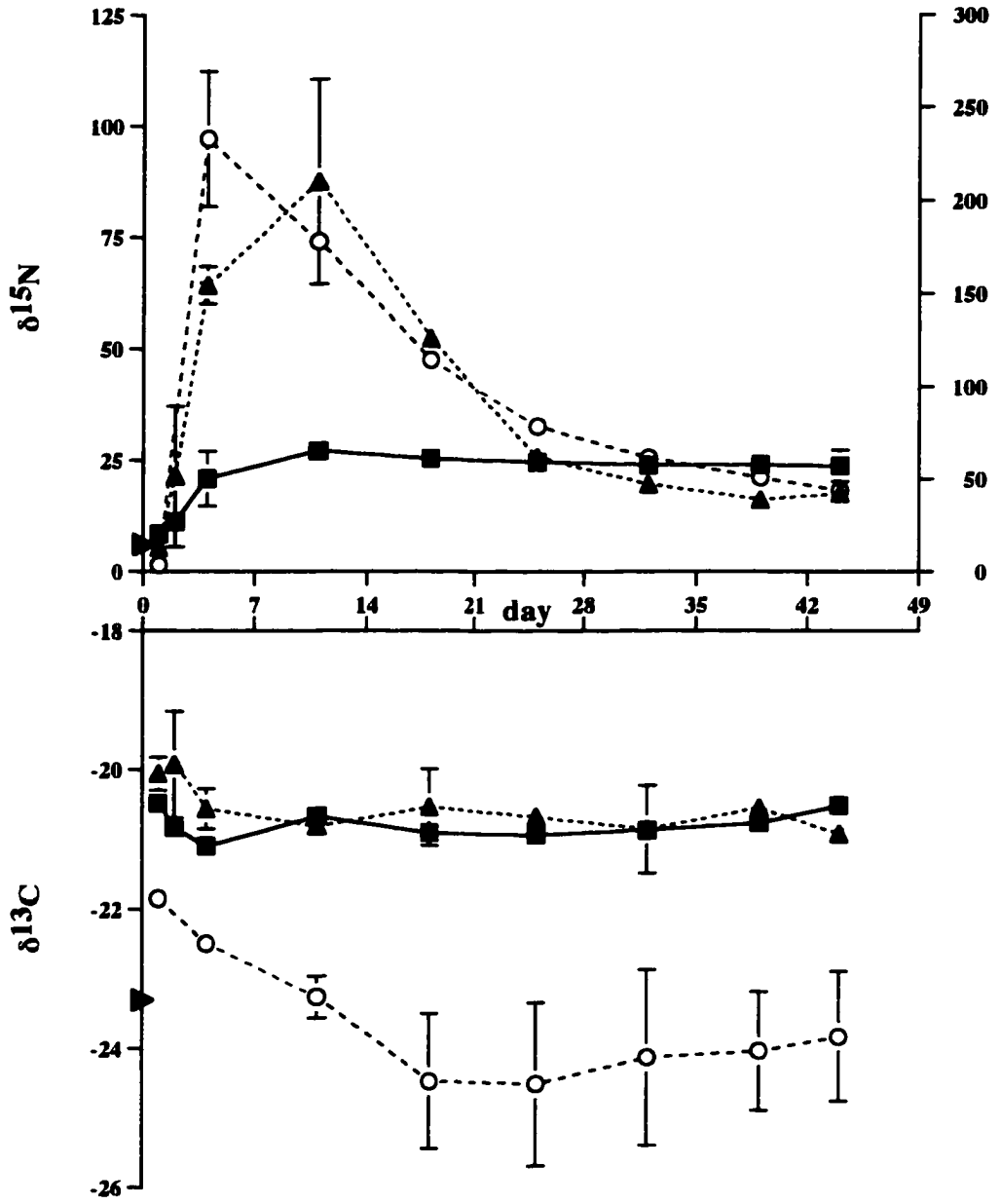

Figure 4.13: $\delta^{15}N$ and $\delta^{13}C$ ratios for green covered OML 99-3 tanks receiving a one time addition of $^{15}NH_4Cl$. N = 2 tanks for muscle, carapace and SPOM each day. SPOM $\delta^{15}N$ values are on secondary y-axis. Error bars represent population standard deviations. ■ muscle; ▲ carapace; O SPOM; ◗ feed.

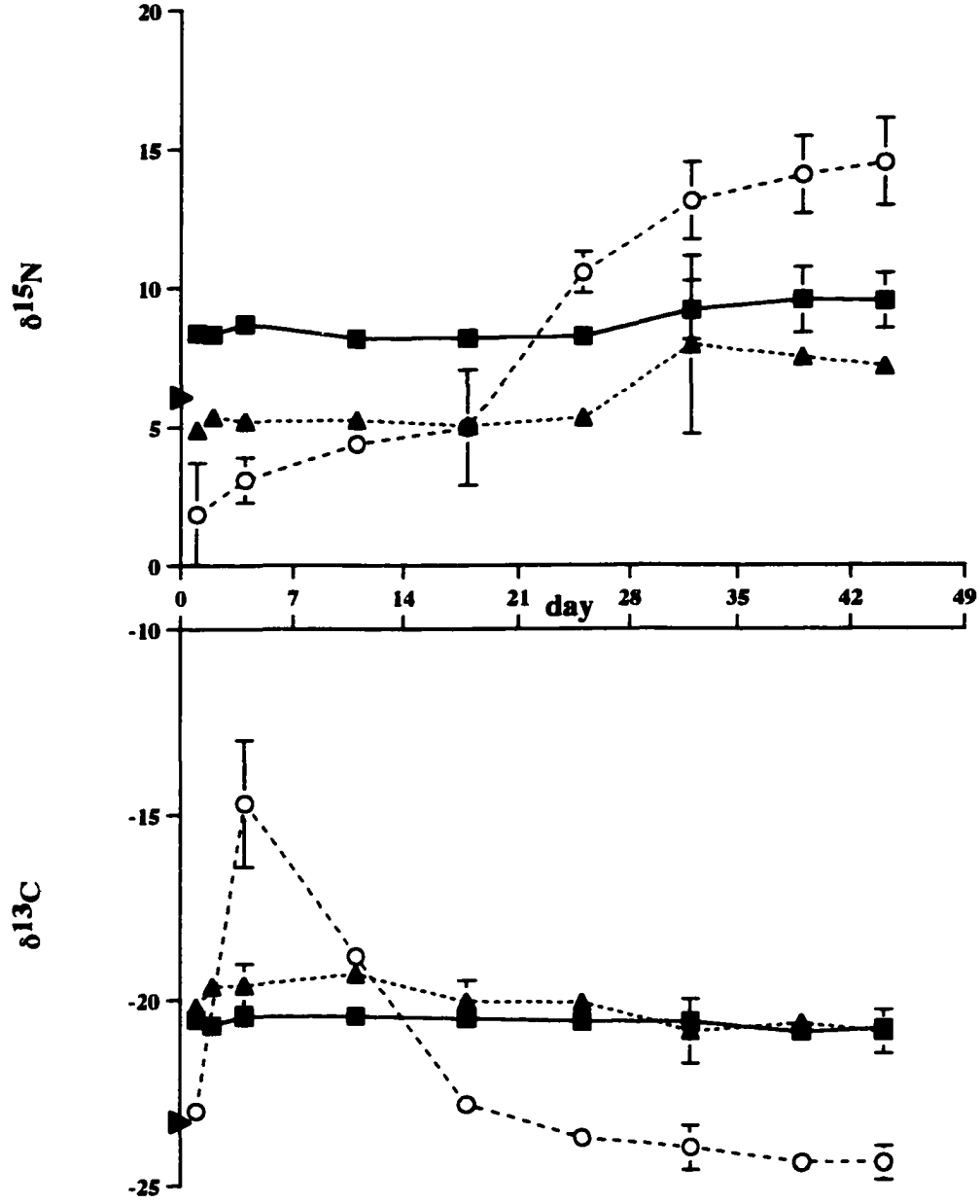

Figure 4.14: $\delta^{15}N$ and $\delta^{13}C$ ratios for green covered OML 99-3 tanks receiving a one time addition of ^{13}C-mannitol. N = 2 tanks for muscle, carapace and SPOM each day. Error bars represent population standard deviations. ■ muscle; ▲ carapace; O SPOM; ◗ feed.

comparisons $p < 0.004$). Statistically there were no differences in the initial slopes between covered and uncovered ammonium addition tanks for any of the sampled components (Figures 4.8 and 4.13).

When mannitol was supplied to the covered tanks (Figure 4.14), the changes in $\delta^{15}N$ and $\delta^{13}C$ over time were similar to those found in the covered control tanks. Furthermore, when the covered and uncovered mannitol additions were compared, there were no statistical differences.

The lack of significant differences, particularly in SPOM values, from covered and uncovered tanks suggested that the green film applied to the tanks was inadequate to limit the photosynthetically active radiation. Visual inspection of the covers four weeks after their application also indicated breakdown of the material due to sun exposure.

OML 01-1

Black covered tanks receiving $^{15}NH_4Cl$ in OML 01-1 (Figure 4.15) showed similar carapace, muscle, and SPOM $\delta^{15}N$ values as the uncovered $^{15}NH_4Cl$ tanks from the same experiment (Figure 4.9). Maximal $\delta^{15}N$ values for SPOM and shrimp muscle were similar to those found in the uncovered $^{15}NH_4Cl$ addition tanks (SPOM = 11518.0 ± 306.7‰ in uncovered, 11369.6 ± 35.4‰ in black covered; muscle = 980.3 ± 64.6‰

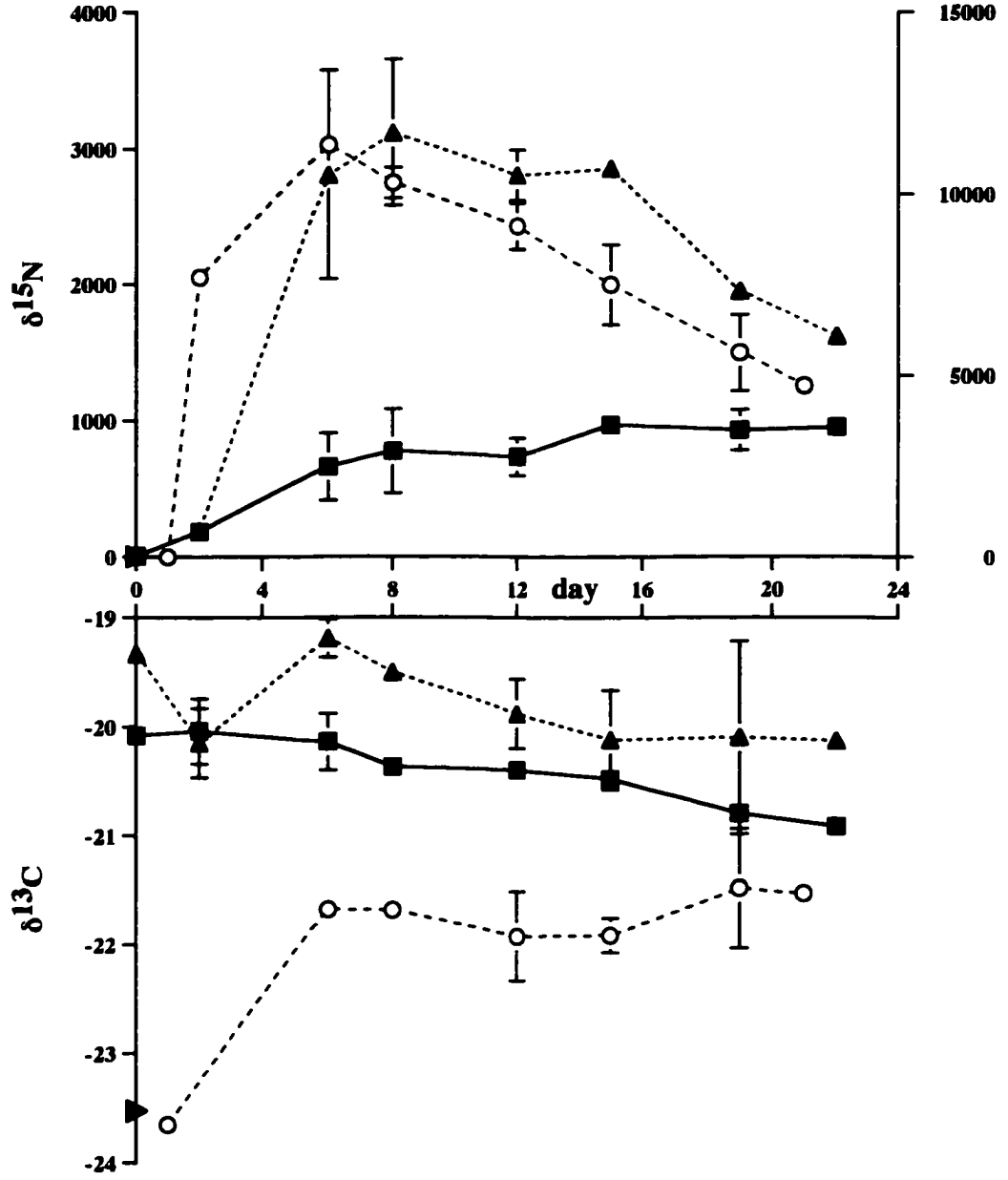

Figure 4.15: $\delta^{15}N$ and $\delta^{13}C$ ratios for black covered OML 01-1 tanks receiving a one time addition of $^{15}NH_4Cl$. N = 2 tanks for muscle, carapace and SPOM each day. SPOM $\delta^{15}N$ values are on secondary y-axis. Error bars represent population standard deviations. ■ muscle; ▲ carapace; O SPOM; ▸ feed.

for uncovered, 974.0 ± 33.8‰ for black covered). However, the time to reach maximal values in the SPOM was four days later, and in muscle seven days earlier, than in uncovered tanks. Carapace $\delta^{15}N$ values for the black tanks actually reached higher maximal values than those in uncovered tanks (3117.9 ± 535.3‰ vs. 1750.6 ± 365.9‰ in the uncovered tanks). Comparisons of the slopes for the change in muscle and carapace $\delta^{15}N$ over time (up to their maximal values) indicated no difference between uncovered and black covered tanks. The rate of increase in SPOM $\delta^{15}N$ to its maximal value was significantly different from that in the uncovered tanks (slope analysis; overall $p < 0.05$, individual comparisons $p < 0.003$). Muscle and carapace $\delta^{13}C$ results were similar to those of the control tanks and uncovered $^{15}NH_4Cl$ tanks (Figures 4.9 and 4.10), but SPOM $\delta^{13}C$ leveled off at –21.5‰ rather than increasing to –18‰ as in the other 2 treatments. Chlorophyll *a* measurements indicated the effectiveness of the black covers in limiting algal growth (pers. comm. L. Conquest, Figure 4.16).

Percent Label Reflected in Muscle

As with the feed experiments, percent of available label was calculated for the pulse additions in order to correct for the different maximum values found among the experiments (Tables 4.3 & 4.4). The direct addition of $^{15}NH_4Cl$ to the water in OML 98-1 resulted in the appearance of 11.4 ± 4.2% of the available label in the shrimp muscle

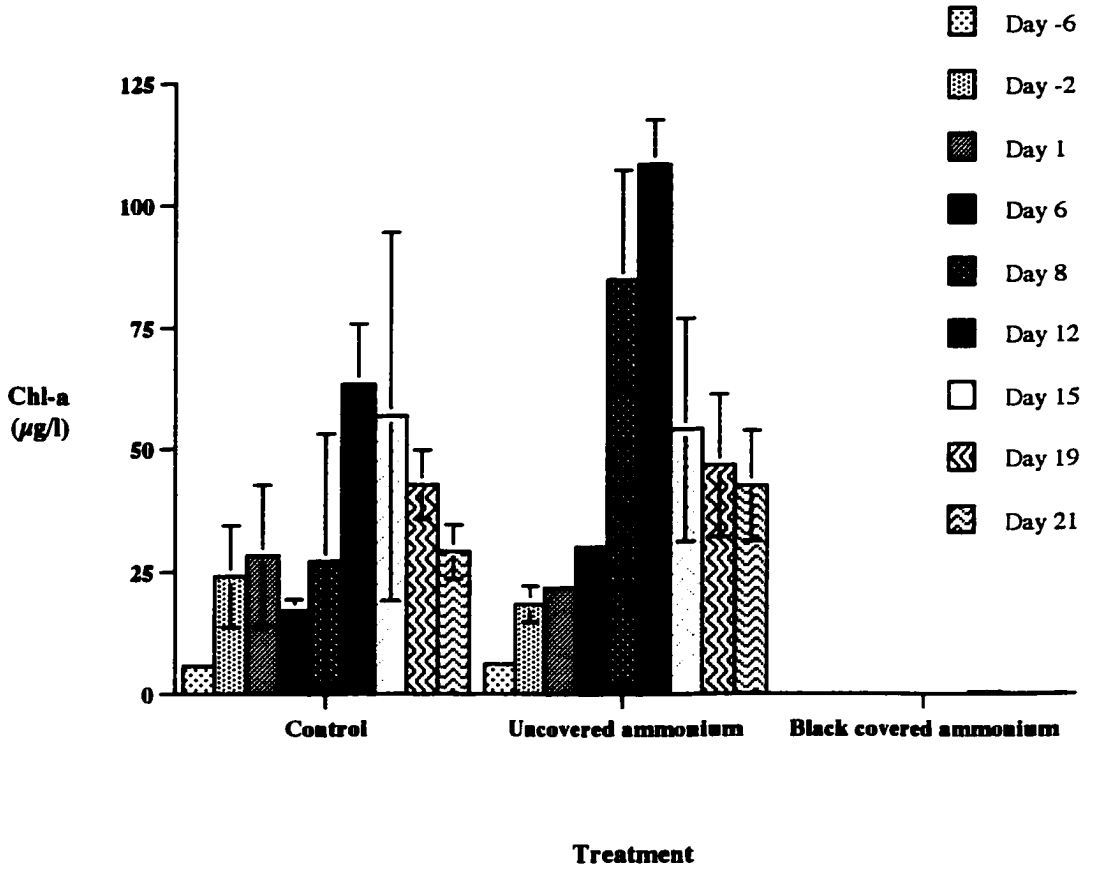

Figure 4.16: Chlorophyll *a* profiles for OML 01-1. N = 2 tanks for each day. Error bars represent population standard deviations.

Table 4.3: Percent of available label found in shrimp muscle tissue for $^{15}NH_4Cl$ additions.

Day	OML 98-1	Day	OML 98-5	Day	OML 99-3		Day	OML 01-1	
					uncovered	covered		uncovered	covered
8	5.30 (2.78)	6	18.2 (7.8)	4	3.44 (4.33)	3.28 (1.54)	6	5.20 (2.13)	5.30 (2.14)
15	7.93 (1.24)	13	23.5 (2.9)	11	8.52 (7.78)	5.85 (0.21)	8	5.66 (0.09)	6.82 (2.96)
22	12.4 (1.4)	20	23.5 (2.3)	18	7.56 (5.31)	6.76 (0.56)	12	10.2 (3.8)	7.50 (1.74)
29	13.9 (3.9)	27	25.4 (2.8)	25	9.54 (7.16)	7.68 (0.18)	15	11.5 (1.3)	11.0 (0.2)
36	13.8 (1.1)	34	29.2 (2.3)	32	9.36 (6.41)	8.42 (0.29)	19	14.0 (0.5)	12.0 (2.6)
43	15.0 (3.4)	41	30.4 (2.0)	38	8.67 (5.20)	9.54 (0.44)	22	15.6 (0.8)	13.2 (0.9)
		48	26.2 (3.4)	44	10.1 (6.6)	10.4 (1.7)			
		55	30.7 (3.9)						
		62	33.3 (4.0)						
Overall									
	11.4 (4.2)		27.0 (5.1)		8.17 (5.04)	7.42 (2.40)		10.4 (4.3)	9.31 (3.38)

Numbers in () represent tank standard deviations.
n=3 tanks for each daily value for OML 98-1 & OML 98-5, N=2 tanks for OML 99-3 & OML 01-1.
Overall values represent the average of all tank daily values for each treatment.

Table 4.4: Percent of available label in shrimp muscle tissue for ^{13}C-mannitol additions.

Day	OML 99-3	
	uncovered	covered
4	-0.03 (0.13)	0.20 (0.04)
11	0.05 (0.05)	0.63 (0.07)
18	0.40 (0.15)	0.81 (0.05)
25	0.62 (0.15)	0.75 (0.23)
32	0.07 (0.26)	1.03 (0.24)
38	0.37 (0.41)	0.33 (0.28)
44	0.32 (0.73)	0.19 (0.40)
Overall		
	0.26 (0.34)	0.56 (0.36)

Numbers in () represent tank standard deviations.
n = 2 tanks for each daily value.
Overall values represent the average of all tank daily values for each treatment.

over the course of the experiment (Table 4.3). The $^{15}NH_4Cl$ addition in OML 98-5 resulted in even higher values with 27.0 ± 5.1% of the label appearing in the shrimp muscle. However, in OML 99-3, only of 8.2 ± 5.0% of the available label was present in the shrimp muscle tissue for uncovered tanks and 7.4 ± 2.4% for the tanks with green covers. Percent label values for OML 01-1 were slightly higher than those found in OML 99-3, with 10.4 ± 4.3% for uncovered tanks and 9.3 ± 3.4% for black covered tanks. Statistically, the overall value for OML 98-5 was significantly higher than those of the other trials (one-way ANOVA combined with Tukey procedure; $p < 0.001$). When values obtained approximately 3 weeks after addition were compared, OML 98-5 showed higher uptake rates by the shrimp than any of the other trials. Shrimp percent label values from covered tanks in OML 99-3 were statistically lower than those in uncovered tanks from OML 01-1.

Label incorporation was essentially zero in the tanks receiving ^{13}C-mannitol (0.2 ± 0.4% for uncovered tanks and 0.7 ± 0.4% for covered tanks). Statistically, shrimp in the covered and uncovered tanks had percent label values that were not significantly different.

Contribution of Natural Tank Populations to Shrimp Growth

Using the information from the $^{15}NH_4Cl$ experiment, it was possible to estimate

the relative contributions of tank natural production to the nitrogen used in growth by the shrimp for each experiment (Table 4.5). As expected, the patterns in percent label values matched those seen for percent contribution of tank natural production. Over the course of the OML 98-1 trial, tank natural production accounted for an average of 28.1 ± 7.0% of shrimp growth nitrogen. The contribution in OML 98-5 was much higher than that in OML 98-1, with daily averages ranging from 41.8 to 76.7% over the experiment. Natural production accounted for an average of 53.6% ± 11.4% of shrimp growth over the 62 days of the experiment. Daily averages in OML 99-3 ranged from 17.0 to 37.6%. Overall, natural production accounted for 23.2% ± 16.4% of shrimp growth nitrogen in uncovered tanks and 23.0 ± 4.7% in green covered tanks. The large standard deviation associated with the uncovered average was due to great differences found in two individual tanks used for that trial: natural production in tank 4-12 accounted for 36.1 ± 13.7% and tank 5-5 had 10.3 ± 1.1%. Tank natural production represented 21.5 ± 4.2% and 23.2 ± 5.5% of shrimp growth nitrogen for uncovered and black covered tanks, respectively, in OML 01-1.

When compared with other trials, OML 98-5 had a significantly higher contribution by tank natural populations to shrimp growth (one-way ANOVA combined with Tukey procedure; $p < 0.001$). To adjust for the differing durations of the

Table 4.5: Contribution of natural production to shrimp growth nitrogen in $^{15}NH_4Cl$ addition tanks.

Day	OML 98-1	Day	OML 98-5	Day	OML 99-3		Day	OML 01-1	
					Uncovered	Covered		Uncovered	Covered
8	26.8 (14.9)	13	76.7 (4.8)	11	37.6 (35.7)	31.8 (0.8)	6	20.78 (8.6)	29.5 (8.6)
15	24.2 (4.4)	20	57.7 (8.1)	18	22.1 (16.3)	22.7 (0.1)	8	17.3 (0.4)	26.3 (8.9)
22	30.9 (3.4)	27	52.9 (7.6)	25	23.3 (18.4)	20.6 (1.2)	12	22.8 (8.1)	18.8 (2.8)
29	30.2 (7.4)	34	54.7 (4.2)	32	20.0 (14.6)	20.2 (2.6)	15	21.9 (2.1)	22.7 (1.2)
36	27.7 (1.0)	41	52.1 (0.7)	39	17.0 (11.1)	21.2 (2.8)	19	22.7 (0.8)	20.6 (3.5)
43	28.6 (8.4)	48	41.8 (8.0)	43	18.9 (13.2)	21.5 (5.4)	22	23.3 (0.9)	21.0 (0.6)
		55	45.8 (6.8)						
		62	47.4 (6.3)						
Overall	28.1 (7.0)		53.6 (11.4)		23.2 (16.4)	23.0 (4.7)		21.5 (4.2)	23.2 (5.5)

Numbers in ( ) are standard deviations
n = 3 tanks for OMLs 98-1 & 98-5, n = 2 tanks for OMLs 99-3 & 01-1.

experiments, the contribution of tank natural production to shrimp growth was compared a second time using only values from approximately 3 weeks after the label addition. The results for the limited time period were the same.

DISCUSSION

The data from the $^{15}NH_4^+$ additions clearly indicate the importance of tank biota to shrimp growth, with 17 to 77% of the nitrogen required for muscle protein being derived from natural production. These values were similar to those found in previous studies of shrimp in semi-intensive pond cultures, in which 52% of shrimp nitrogen was from pond biota (Parker et al., 1989). However, most studies of this type have been performed using carbon isotopes. For comparative purposes, it is estimated (based on a SPOM C:N ratio of 5.5) that an average of 50% of the shrimp's carbon growth is due to natural production. This compares well with values found by other researchers. Anderson et al. (1987) and Parker et al. (1989) concluded that 44 to 86% of *P. vannamei* growth carbon was from pond biota. Nunes et al. (1997) found similar values for *P. subtilis*, 75% of growth C. Using gut content analysis, Focken et al. (1998) determined that pelleted feeds in pond culture systems accounted for no more than 50% of *P. monodon* diets. However, they noted that formulated feed might have been underrepresented in shrimp stomachs due to better digestibility (Focken et al., 1998).

While the results presented here are similar, it is important to note that this study is not truly comparable to previous studies, which were undertaken in semi-intensive culture ponds with stocking densities much lower than used here (Table 4.6).

Given the toxicity of ammonium to shrimp, it was necessary to be concerned about increasing the background nutrient concentrations with negative effects on the shrimp (Alcaraz et al., 1999b; Frías-Espericueta et al., 1999).The values found in these tanks were less than maximal values found in other trials. Median lethal concentrations for *Penaeus chinensis* are 41 mg/l for ammonia-N and 117 mg/l for nitrite-N after 72 hours exposure (Chen et al., 1990). Lethal concentrations for *Penaeus setiferus* are 8.69 mg/l and 167.33 mg/l, respectively, after 72 hours exposure (Alcaraz et al., 1999a). The concentrations in these trials were much lower and should not have adversely affected the shrimp.

The varying results for the contribution of natural production among the 4 trials are difficult to explain. One possible explanation would be different growth rates for the shrimp resulting in dissimilar uptake of the label. However, while growth rates were lower in OMLs 98-1 and 01-1 than the other trials, OML 98-5 and 99-3 had similar growth rates, yet the OML 98-5 tank natural contribution values are considerably higher than OML 99-3 values. Several researchers have noted that the diets of wild shrimp

Table 4.6: Comparison of experimental conditions for published reports of contribution of natural production to shrimp growth.

Species	Method	% growth from natural assemblage	Stocking weight (g)	Density (#/m^2)	Growth (g/wk)	Reference
Penaeus vannamei	$\delta^{13}C$	44-86	1.5	20	0.7-0.95	Anderson et al., 1987 Parker et al., 1989
Penaeus vannamei	$\delta^{15}N$	52	1.5	20	0.7-0.95	Parker et al., 1989
Penaeus subtilis	$\delta^{13}C$	75	1.39	10	1.36	Nunes et al., 1997
Penaeus monodon	Gut content	50	0.35	7	0.91	Focken et al., 1998
Litopenaeus vannamei	$\delta^{15}N$	17-71	0.78–3.63	50-56	0.45–1.87	This study

change as they increase in size (Nunes and Parsons, 1998; Stoner and Zimmerman, 1988). Smaller shrimp utilize more algae, with a switch to animal protein as the shrimp grow (Robertson, 1988; Rothlisberg, 1998). However, shrimp in OML 98-5 were larger than in each of the other trials at the time of the label addition. The number of shrimp in the tanks could have also been a factor, as more shrimp would increase competition for food resources and the need to utilize more of the added formulated feeds. Parker et al. (1989) indicated that at low shrimp densities, natural production may be enough to support shrimp growth, limiting the need for added feeds. Contrary to expectation, the contribution in OML 98-1 (tanks having the highest survival and thus high shrimp density) was less than that in OML 98-5 (average survival of 38%), but greater than that in OMLs 99-3 and 01-1 (average survival of 60.3% and 37.3% respectively). Higher shrimp densities did not lead to increased reliance on formulated feed, given that the tank contribution values were similar for all trials except OML 98-5. The composition of the feeds differed slightly among trials, with crude protein ranging from 30.8% to 39.4% and crude lipid varying from 8.7% to 9.2% (see Appendix D). However, shrimp from OML 98-1 and 98-5 were provided the same feeds, yet had statistically different relative contributions. Therefore, the differences in feed formulation were probably not great enough to result in such large variations in feed utilization values. Another factor

that could have contributed to reported differences is the composition of the algal community during the study. Moss (1994) found that juvenile *Penaeus vannamei* fed green algae and macroalgae did not grow as well as those fed diatoms. While species composition was not monitored in the first three trials, changes from small green algae to larger diatoms were noted in OML 01-1. Therefore, the differences among the trials are likely due to a combination of environmental factors and tank biota that affect shrimp growth.

Tests to determine the extent of indirect versus direct contributions of tank biota to shrimp biomass were inconclusive. In previous studies, Leber and Pruder (1988) suggested that the improved growth of shrimp in the presence of natural production might be the result of growth factors present in natural pond water. Rubright et al. (1981) indicated that the increased growth of *Penaeus stylirostris* in the presence of natural algal populations occurred after these natural populations entered the benthic food web. The large fraction of isotope label evident in the shrimp indicates that the contribution of algae to shrimp growth nitrogen was due to direct consumption. This is supported by epifluorescence microscopy, which indicated the presence of algal cells in shrimp guts (O. Decamp, pers. comm.) as well as previous studies (Bombeo-Tuburan et al., 1993; Hunter et al., 1987; Mann, 1988; Moss, 1994).

The most interesting finding of this study is the similarity in results for the uncovered and black covered NH_4^+ addition treatments of OML 01-1. Given the presence of algae in shrimp guts and the assumed direct linkage, it was postulated that the amount of label that would make its way into shrimp tissues would be small in the black covered tanks compared to uncovered tanks. One possible mechanism to account for the large amount of label appearing in the shrimp in black tanks is that the shrimp are removing $^{15}NH_4^+$ directly from the water. Ammonia could be actively transported across gill membranes and then metabolized for the production of chitin (Smucker, 1991) and to a lesser extent the synthesis of amino acids. The old carapace is partially resorbed by the shrimp when they next molt and many of the proteins and minerals are retained for the production of the next carapace (Lee and Wickins, 1992). While it seems unlikely that the shrimp would obtain enough label via this ammonium uptake to account for the $\delta^{15}N$ values found here, it is an aspect that warrants more study.

Another possibility considered was that isotopically labeled bacteria were attaching themselves to the shrimp carapace and, when the carapace is resorbed, the label transferred into the muscle tissue. Carapace samples were analyzed before and after scrubbing to remove any attached bacteria and indicated no significant differences. Therefore, attached bacteria have a small mass compared to the carapace and do not

affect the isotope ratio.

A more likely pathway for the appearance of the label in the shrimp in dark tanks is via bacterial production. It is known that bacteria are able to utilize ammonia (Burrell et al., 2001; Goldman and Dennett, 2001; McCraig et al., 1999) and that bacteria are found in the gut contents of shrimp (Moss et al., 2000; Robertson, 1988). However, the contribution of bacteria to shrimp growth is generally unknown (Bombeo-Tuburan et al., 1993). Bacteria clearly play a role in nutrient recycling in these ponds and also likely act as a food source for the shrimp either through direct utilization or consumption of meiofauna that consume bacterial production (Moriarty, 1997).

Past research has suggested direct utilization of bacteria by shrimp. Moriarty (1976) found bacteria in the proventriculus of *Metapenaeus bennettae* in quantities greater than would be found for symbiotic bacterial assemblages. Further examination of a variety of Penaeid species supported this finding, with 10 to 20% of the material in the proventriculus comprised of bacteria (Moriarty, 1977). Furthermore, he noted only limited amounts of algal material in each of these studies. However, with improved assay techniques, Moriarty and Barclay (1981) concluded that earlier results were likely overestimates of the bacterial contribution. Still, they documented that, while bacteria constituted only 2% of the organic matter found for adult *Penaeus merguiensis*, up to

14% of the organic carbon in juvenile shrimp foreguts was from bacteria. Langdon and Newell (1990) found that bacteria could provide 20 to 70% of bivalves' nitrogen requirements.

Even though bacteria are known to play an important role in the nutrient cycling in these aquaculture systems, most researchers discount a large role for bacteria in direct shrimp nutrition. Studies examining the energetics of feeding directly on bacteria suggested that detritivores would be unable to sustain themselves on bacterial biomass alone (Blum et al., 1988; Cammen, 1980). Moss et al. (1992) found that particles greater than 0.5 μm, primarily diatoms and aggregates of microbes and detritus, were responsible for the growth enhancement of shrimp in intensive culture conditions. However, they were unable to attribute the enhanced growth to a feeding enhancer or direct utilization. Moss and Pruder (1995) estimated the bacterial biomass available to shrimp in these systems and suggested that suspended bacteria were of little importance in shrimp diets.

The finding here that 23% of the nitrogen required for shrimp growth is derived from bacterial sources is similar to the findings of the studies above. The bacterial contribution is likely a result of uptake of ammonia by bacterial populations, concentration of these populations through chain formation or attachment to detrital

particles, followed by direct consumption of these by the shrimp. An important point to note is that the contribution value found here is not representative of the natural system and likely overestimates the bacterial contribution due to the manipulated environment (i.e. black covers inhibiting algal growth). The addition of ^{13}C-labeled mannitol to the tanks would have provided more information on this linkage, but the added amount was insufficient relative to the background pool of carbon. If the added label had been quantitatively taken up by the particulate organic carbon pool, this pool would have been enriched approximately 30‰. However, the SPOM pool only showed 5‰ enrichment, likely indicating a large pool of unlabeled carbon as well as efficient respiration of the label. The results presented here clearly indicate the linkage between bacteria and shrimp and as well as the need for future studies of this nutrient pathway. Future studies should make use of multiple isotope techniques to separate the role of algal versus bacterial production to shrimp growth.

CONCLUSIONS

The addition of labeled ammonium to uncovered tanks indicates that natural microfloral production is contributing between 17 and 77% to shrimp growth nitrogen. Variations among the results of the experiments were likely due to a combination of environmental factors and differing tank biota compositions. An initial attempt to assess

bacterial production via the addition of labeled mannitol to uncovered tanks was unsuccessful due to insufficient label. However, the addition of labeled ammonium to black covered tanks resulted in similar contribution values, suggesting that bacteria play an important role in shrimp nutrition. It is suggested that further experiments include double labeling techniques to compare bacterial and algal contributions.

CHAPTER 5 – ESSENTIAL AMINO ACIDS PROVIDED BY TANK NATURAL PRODUCTION

INTRODUCTION

Amino acids from diet are important for the production of new proteins and as a source of energy to the consumer (Goddard, 1996). Studies on shrimp using radiolabel incorporation (Cowey and Forster, 1971; Kanazawa and Teshima, 1981) have given useful information on the amino acid profiles, with particular reference to essential amino acids. The essential amino acids that the shrimp either do not produce or produce at low levels have been identified as arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, and valine (Akiyama et al., 1992; Cowey and Forster, 1971; Goddard, 1996; Kanazawa and Teshima, 1981). The provision of essential amino acids in a correct balance in shrimp aquaculture is important when attempting to create a complete feed. The usual approach for determining the amounts of each of the essentials to include is to provide a feed with an amino acid profile which mimics the profile in the shrimp muscle tissue (Peñaflorida, 1989). Deshimaru and Shigeno (1972) found that *Penaeus japonicus* had the highest feeding efficiency on protein sources having similar amino acid profiles as the shrimp.

This method has been widely adopted, but it is agreed that the essential amino acid requirements of shrimp need further investigation (Shiau, 1998).

As indicated in the previous chapter, it is well known that pond biota contribute to shrimp growth (Anderson et al., 1987; Leber and Pruder, 1988; Moss and Pruder, 1995; Moss et al., 1992; Nunes et al., 1997; Parker et al., 1989; Rubright et al., 1981; Schroeder et al., 1984; Schroeder, 1983a; Tacon and Akiyama, 1997). Because shrimp can obtain 50% or more of their growth carbon from the tank production (Anderson et al., 1987; Nunes and Parsons, 1998; Parker et al., 1989), it is unlikely that formulated feeds are fully satisfying their requirements. One possibility for the improved growth in the presence of tank biota is that the biota provides an essential micro-nutrient to the shrimp. Shrimp samples that were isotopically labeled by tank biota provided an opportunity to examine labeling of individual amino acids of the shrimp. Berthold et al (1991) used incorporation of stable isotopically labeled algae to give insight into essential amino acids in hens. The results presented here are an initial estimation of the contribution of tank biota to shrimp amino acids. Stable isotopic tracers were used to track nitrogen from tank biota into individual shrimp amino acids with the anticipation that the pattern of labeling would give some insight into the linkage between tank natural populations and the acquisition of essential amino acids by shrimp.

MATERIALS AND METHODS

Samples

Shrimp from experiments in which labeled ^{15}N-ammonium chloride was added directly to tanks in trials OML 99-3 and OML 01-1 (see Chapter 4 for a detailed description) were used for assessing the appearance of label in individual amino acids. Natural tank biota was isotopically labeled via the addition of ^{15}N-ammonium chloride (99% ^{15}N atom enriched, 0.1 g/tank in OML 99-3 and 0.83 g/tank in OML 01-1). This label was then tracked into individual amino acids in shrimp muscle tissue. Growth rates for the shrimp varied considerably from 0.5, 0.7, and 0.6 g/wk for the black covered, open ammonium, and control tanks in OML 01-1 to 1.7 for control and ammonium tanks in OML 99-3 (see Table 4.2 for growth and efficiency measures). For OML 99-3, two shrimp from tanks 4-6 (control) and 4-12 (ammonium addition) were sampled for amino acid abundance and stable isotopic values for days 1, 2, 11, and 18. In OML 01-1, two shrimp were taken from tanks 5-1 (control), 4-4 (open ammonium addition), and 4-8 (black covered ammonium addition). The shrimp muscle tissue was dried either by oven drying at 80°C for 48 hours or by freeze-drying overnight. The dried tissues were ground to a homogeneous powder and stored until amino acid analysis.

Sample Preparation

Approximately 20 mg of dried muscle tissue was hydrolyzed in 2 ml 6N HCl at 110°C for 22 hours. Samples were flushed for 2 minutes under a stream of N_2 prior to closing the screw cap tubes (Pyrex brand with fluorocarbon resin faced liners, 13 x 100 mm, Fisher cat. # 14-957C). Samples were filtered through a Polypure II syringe (0.2 μm, 13 mm filter, Alltech cat. #6718) after hydrolysis and placed in an oven at low heat (approximately 40°C) until dry.

Amino Acid Concentrations

The separation and identification of amino acids in hydrolyzed shrimp muscle tissue was done using a method modified from Henrichs and Williams (1985), Lindroth and Mopper (1979), and Mopper and Dawson (1986). Dried hydrolyzed samples were brought to 2 ml with deionized water and a 200 μl subsample used for concentration analysis. The remaining 1.8 ml was freeze-dried for subsequent amino acid separation for isotope analysis. Concentrations were determined on HPLC after derivatization with o-phthaldialdehyde reagent (OPA).

The HPLC system used consisted of an Alltech Model 526 binary gradient system, an Alltech Model 570 autosampler, an Alltech Model 530 column heater, a Linear Model 200 UV/vis detector, and a Spectrum fraction collector. Data were

collected on a Inteva LC data station using TurboChrom4 software with Microsoft Windows 98 through a Perkin Elmer NCI 900 Network Chromatography Interface.

Samples were prepared by combining with 0.025 M borate buffer, pH 10.4 and adding α - aminoadipic acid (an internal standard) prior to the addition of the OPA reagent for derivatization (see Appendix F for detailed technique). The sample was reacted for 2 minutes before injection onto an Alltech Allsphere ODS-2 column (5 μm, 250 mm x 4.6 mm, Alltech cat. #778736). The amino acids were separated at 25°C using a gradient from 85% sodium phosphate buffer (0.025 mM, pH 6.5)/15% methanol to 35% buffer/65% methanol and detected at 340 nm. Peak heights were compared to a standard curve obtained using amino acid standards (Sigma, cat. # AA-S-18). Proline was not detected by this technique. A sample chromatogram is given in Figure 5.1.

The concentrations determined via this method were not representative of the exact concentrations due to losses during processing. Tryptophan is destroyed and a number of other amino acids including threonine and serine may not be quantitatively recovered under the hydrolysis conditions employed (Ozols, 1990). Glutamine is converted to glutamic acid during hydrolysis (Ozols, 1990). Furthermore, each processing step may result in further losses. However, because the main goal of concentration analysis was to estimate the amount of material required for mass

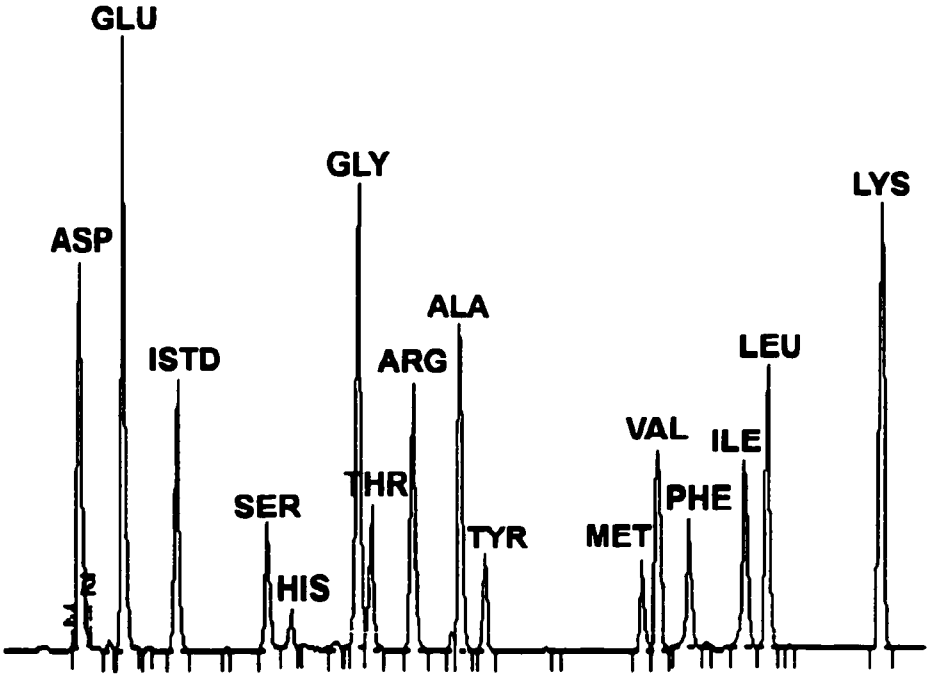

Figure 5.1: Representative chromatogram for OPA separation and quantification of amino acids on Alltech Allsphere ODS-2 column, sample 4-4a, 10 January 2001.

spectrometer analysis, these losses were not disturbing. For comparative purposes, concentrations were represented as relative percentages of the total measured pool.

Amino Acid Stable Isotope Signatures

Isolation of individual amino acids for determination of stable isotope values was performed using a two column technique. Hydrolyzed muscle samples were cleaned by applying them to a solid phase extraction column (SPE, Alltech C18 extract clean column, 500 mg, cat. # 205350) and eluting with deionized water. The eluate was then freeze-dried once more. For the initial amino acid separation, samples were applied to an Alltech Econosphere NH_2 column (5μ, 250 mm x 10 mm, cat. # 28091) at 38°C using a modified technique from Schuster (1980) (see Appendix G). The mobile phases were 0.01 M KH_2PO_4, pH 4.3 and acetonitrile (ACN):water (500:70). Amino acid separation was obtained using a gradient of 10% buffer/90% ACN:H_2O to 50% buffer/50% ACN:H_2O at 4 ml/min (see Appendix G). The underivatized amino acids were detected at 200 nm, and the peak volumes collected and freeze-dried for isotopic analysis. Early separation on this column showed clear peaks for glycine and serine, however as the number of injections onto the column increased glycine and serine co-eluted. When possible, isotope ratios are given separately for the two amino acids as well as a combined ratio for glycine and serine.

Using this technique, it was difficult to adequately separate methionine, isoleucine, leucine, tyrosine, and phenylalanine (Figure 5.2). Therefore, these peaks were combined and applied to an Alltech Alltima C18 column (5μ, 250 mm x 10 mm, cat. # 88063) using a modified method from Wassner and Li (1982) and Hancock and Harding (1984). Amino acids were eluted with ACN:H_20 (500:70) and 0.02 M KH_2PO_4, pH 2.5 using a gradient of 3% ACN:H_2O /97% buffer to 30% ACN:H_2O /70% buffer (Figure 5.3; see Appendix H). Fractions were then collected and dried.

After freeze-drying, individual amino acid fractions were then weighed into tin cups for isotopic analysis. Because recovered methionine, isoleucine, leucine, tyrosine, and phenylalanine concentrations were low in nitrogen, fractions from both shrimp of a single tank were combined to provide enough material for isotopic analysis. Additionally, lysine eluted as a number of peaks, one of which coincided with histidine. Therefore these peaks were combined and given a HIS/LYS designation. Isotope ratios were obtained with a ThermoFinnigan Delta ^{plus} XL mass spectrometer and presented in standard delta notation where: $\delta(‰) = (R_{sample} - R_{std}) / R_{std} \times 1000$. A comparison of stable isotope ratios of amino acid standards before and after injection showed close agreement with differences less than 1‰. Amino acid isotope values for replicate injection and separation of sample protein hydrolysates were generally less than 2%

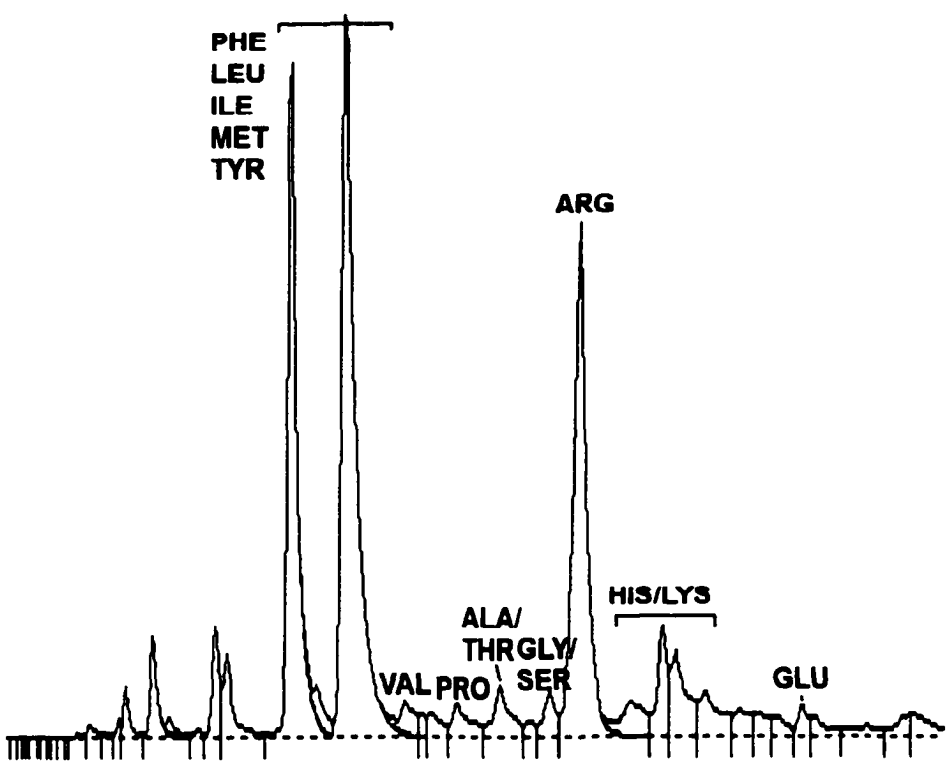

Figure 5.2: Representative chromatogram for separation of underivatized amino acids on Alltech Econosphere NH_2 column, sample 4-4a, 10 January 2001. Detection response factors vary dramatically for different amino acids, so this chromatogram does not correspond to actual composition.

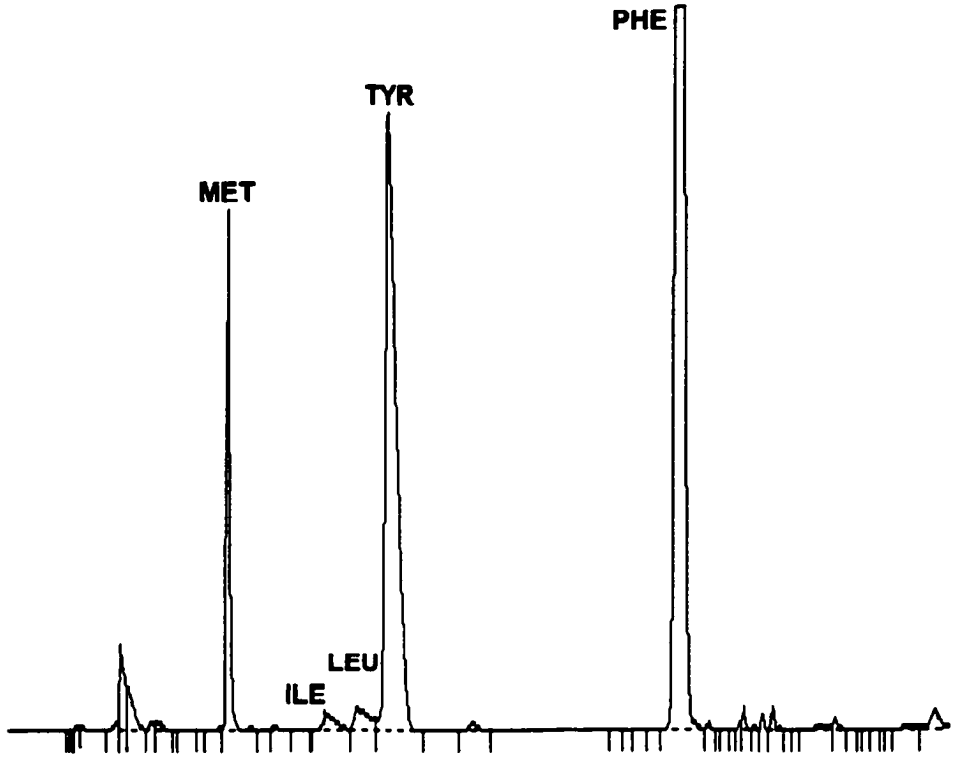

Figure 5.3: Representative chromatogram for separation of MET, ILE, LEU, TYR, and PHE on Alltech Alltima C18 column, sample 4-4a, 10 January 2001. Detection response factors vary dramatically for different amino acids, so this chromatogram does not correspond to actual composition.

different.

Calculations

The contribution of tank biota to shrimp amino acid nitrogen was calculated for each amino acid using the equation

$$\frac{W_p}{W_f} = \frac{\delta_f - \delta_g}{\delta_g - \delta_p}$$

where W_p is shrimp weight gained from pond (in this case, "tank") production, W_f is weight gain from formulated feed and δ_p, δ_f, and δ_g are isotopic ratios for the tank biota, feed, and growth respectively (see Appendix E; Anderson et al., 1987; Parker et al., 1991). It was assumed for this calculation that stable isotope ratios for amino acids in the formulated feeds and tank suspended particulate matter were uniform and whole pool $\delta^{15}N$ values were used in the equation. Given that proline concentrations were not determined by the OPA technique used here, proline calculations were done with an estimate of concentration based on relative concentrations found in other penaeid species (Deshimaru and Shigeno, 1972; Peñaflorida, 1989).

Statistics

The limited number of analyses precluded extensive statistical analyses on the amino acid isotopic information. Concentrations were compared within a trial (i.e. control from OML 99-3 to ammonium tanks from OML 99-3) using the general linear

model (GLM) where day, treatment, and amino acid were used to model concentration (Minitab version 8.21 for Macintosh). GLM analysis was also used to compare the contribution values for the different trials. The increases in $\delta^{15}N$ values for the individual amino acids for experimental versus control tanks were related by comparing their slopes of $\delta^{15}N$ versus day using the following equation (as given in Chapter 2):

$$t = (b_1 - b_2)/SQRT(SE_{b1}^2 + SE_{b2}^2)$$

where b_1 and b_2 are the slopes of the two lines being compared and SE is the standard error of the regression line (Fowler et al., 1998). This comparison was only performed between treatments to satisfy the assumption of independence. The Bonferroni multiple comparisons procedure was used with an overall significance of 0.05 to limit the possibility of Type I error (Mendenhall and Sincich, 1996).

RESULTS

Amino Acid Concentrations

The relative concentrations of the amino acids were similar over time for both OML 99-3 samples and OML 01-1 samples (Figures 5.4 and 5.5 respectively). Overall, glutamic acid represented the largest fraction of amino acid in the hydrolyzed muscle tissue, at approximately 20% of the total examined. The abundance trends in relative concentrations of the amino acids were the same among all tank treatments with

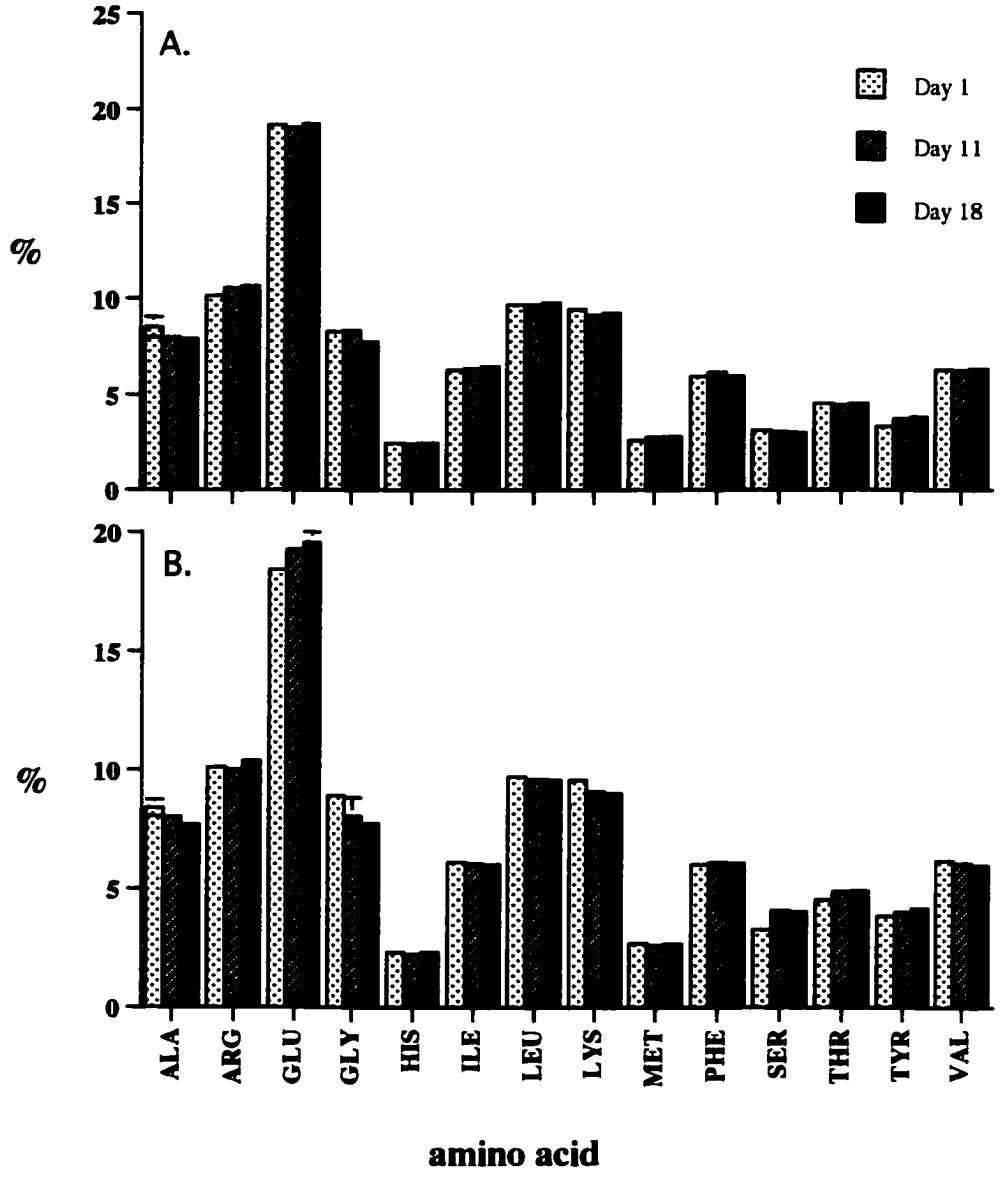

Figure 5.4: Amino acid concentration profiles for shrimp muscle protein, trial OML 99-3. Concentrations are expressed as percentage of the total amino acid pool. A. Control tanks, B. Ammonium tanks. Bars represent range, n = 2 shrimp.

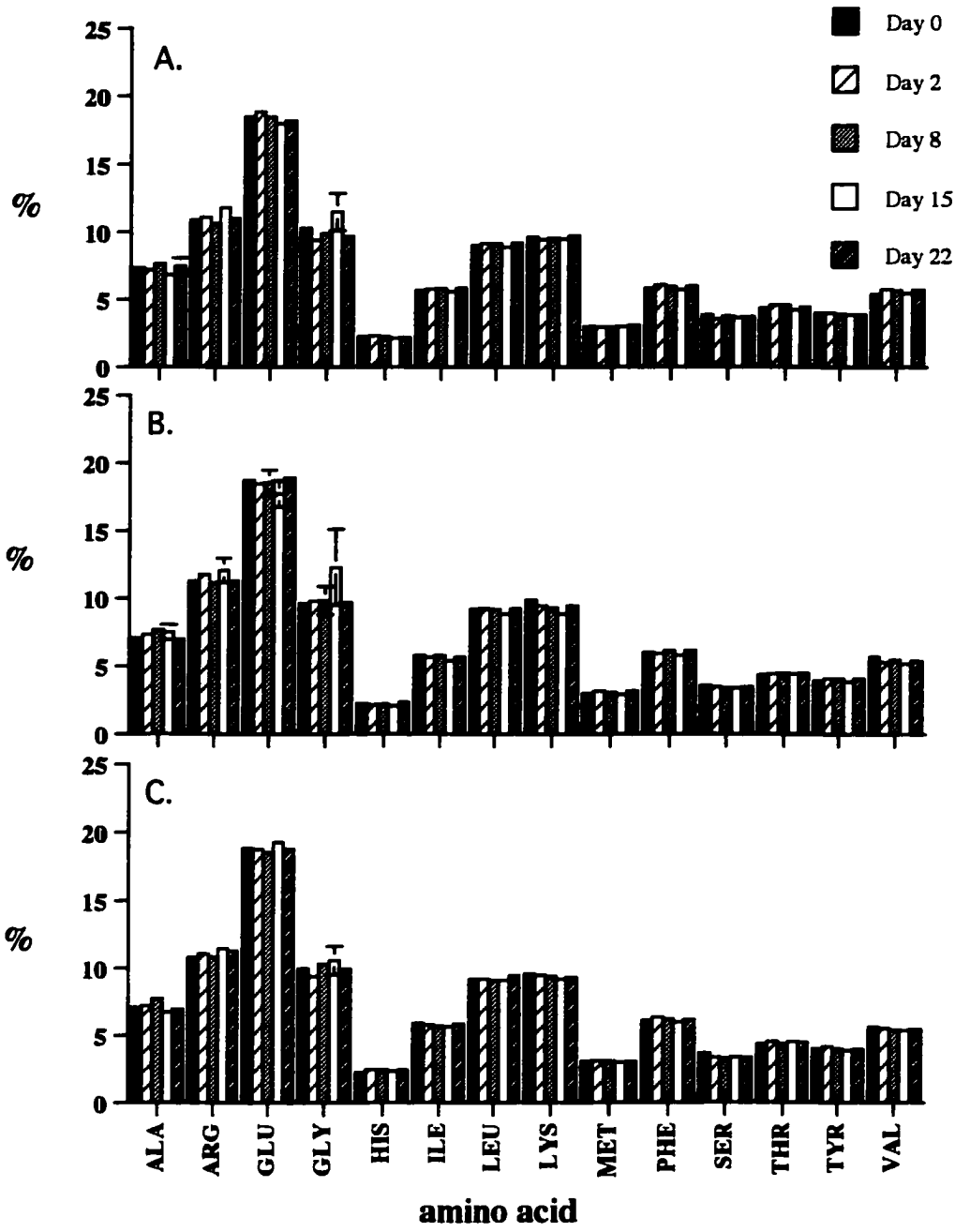

Figure 5.5: Amino acid concentration profiles for shrimp muscle protein, trial OML 01-1. Concentrations are expressed as percentage of the total amino acid pool. A. Control tanks, B. Ammonium tanks, C. Black covered ammonium tanks. Bars represent range, n = 2 shrimp.

differences of less than 1%. When GLM analysis was performed within a given trial, concentrations were not affected by day or treatment. Therefore, the only differences in relative concentrations were between individual amino acids.

Amino Acid Stable Isotope Signatures

The stable isotopic signatures for OML 99-3 samples are given in Figure 5.6. $\delta^{15}N$ values for the amino acids on day 1 were not significantly different between control and ammonium addition tanks (GLM analysis prior to $^{15}NH_4^+$ addition). Additionally, the isotopic signatures for shrimp in the control tanks did not change significantly over time. There were clear increases in $\delta^{15}N$ values for the ammonium tanks as compared to the controls after the addition of the isotopically labeled ammonium. One day after the addition of the isotopic label, $\delta^{15}N$ values for amino acids in the ammonium addition tanks were higher than those in control tanks for all amino acids except leucine (methionine concentrations for control samples on day 1 and day 2 were too small for mass spectrometer analysis). After one day, methionine and alanine/threonine had the largest increases in $\delta^{15}N$, when compared to the values prior to addition, with a difference of 22‰ and 17‰, respectively. All of the amino acids in the ammonium tanks had similarly elevated $\delta^{15}N$ values, except glutamic acid and histidine/lysine by day 11. When the slopes of the amino acid $\delta^{15}N$ values versus day

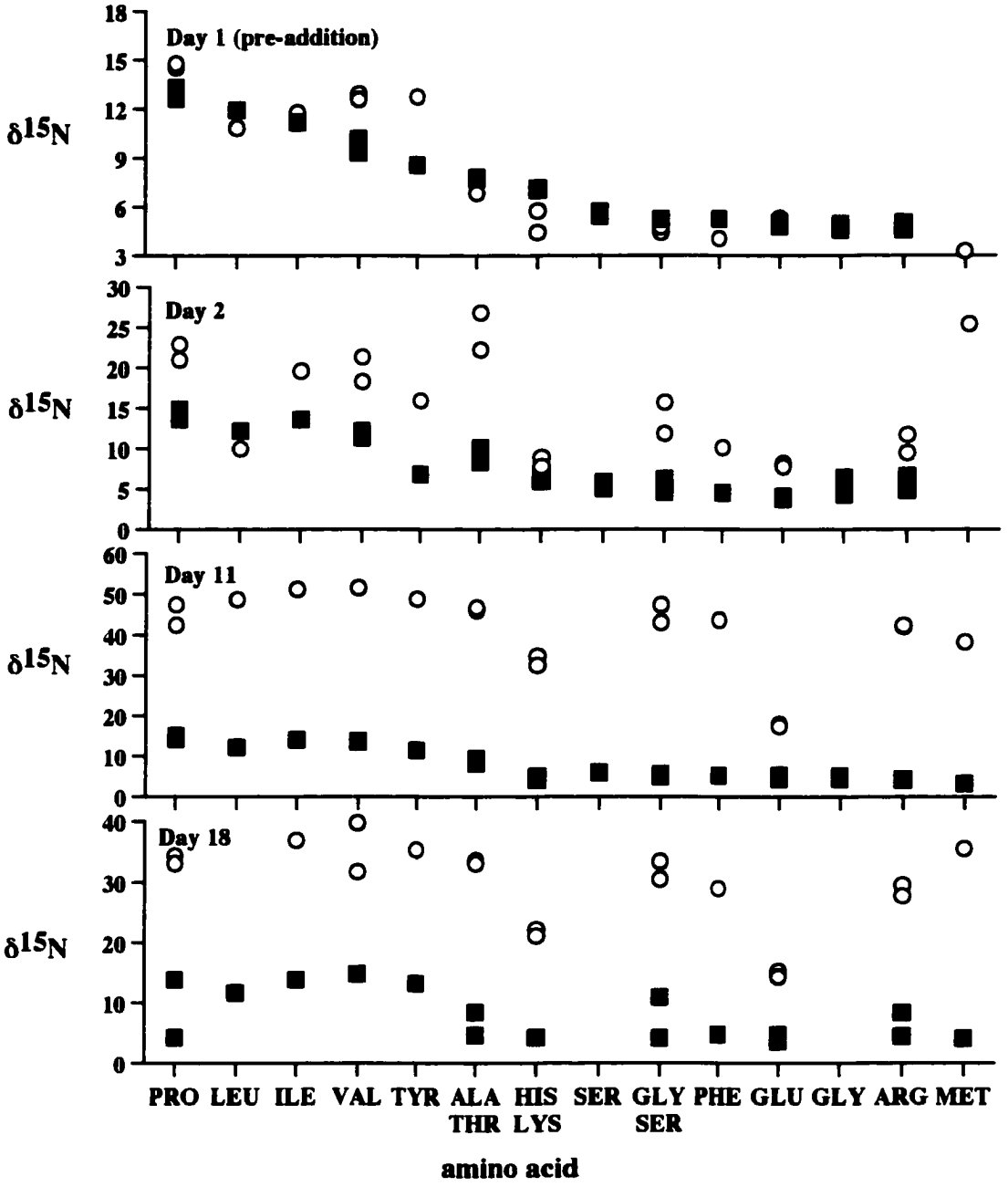

Figure 5.6: $\delta^{15}N$ values for OML 99-3 shrimp muscle in tanks receiving a one time addition of $^{15}NH_4Cl$. ■ control tanks; O ammonium tanks. Glycine and serine ratios are given separately, when clear separation was obtained, in addition to combined values.

were compared out to day 11, all were significantly greater than their respective control values except isoleucine, leucine, and methionine, probably due to limited samples for these amino acids (slope analysis; overall $p < 0.05$, individual comparisons $p < 0.004$). On day 18, the $\delta^{15}N$ values for each of the amino acids have declined from day 11 values as the label is diluted by unlabeled nitrogen in the growing shrimp.

As with OML 99-3 samples, the $\delta^{15}N$ values for the individual amino acids on day 0 were similar for all of the treatments in OML 01-1 (Figure 5.7). While the addition of labeled ammonium caused marked increases in both the open and black covered tanks, the $\delta^{15}N$ values for the control tanks were not significantly different over time. Day 2 values for all amino acids in the open ammonium tanks were higher than control values. The largest differences in $\delta^{15}N$ between day 0 and day 2 values in ammonium addition tanks were for proline (67‰) and alanine/threonine (29‰). On day 8, the amino acids in the open addition tanks had sharply increased yet variable $\delta^{15}N$ values, with large differences among individual shrimp. Alanine/threonine, arginine, proline, glycine/serine, and valine had individual higher values than the other amino acids, although slope analysis indicated significant differences between control and open ammonium tanks for alanine/threonine and glycine/serine only (slope analysis; overall $p < 0.05$, individual comparisons $p < 0.002$).

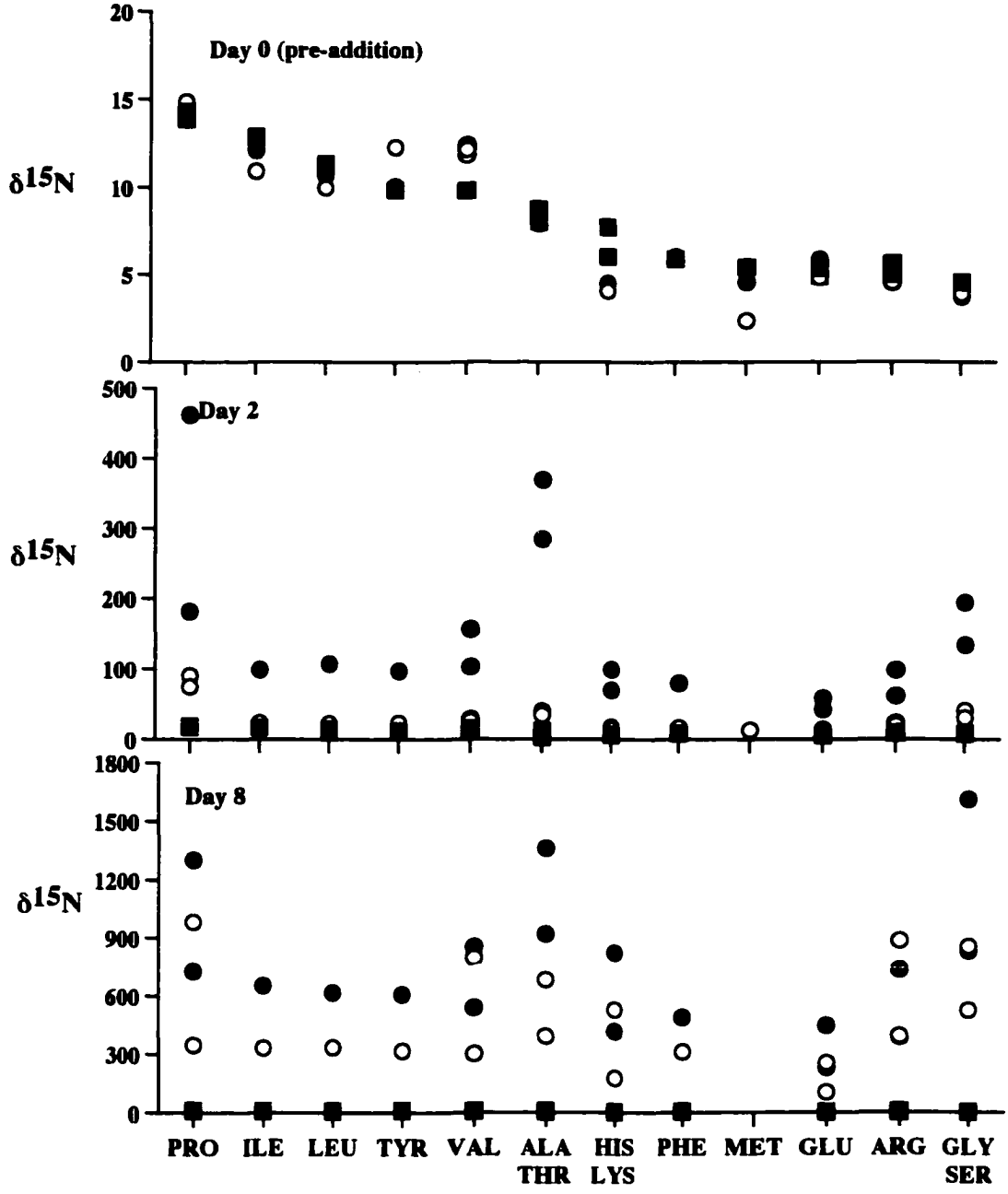

Figure 5.7: $\delta^{15}N$ values for OML 01-1 shrimp muscle in tanks receiving a one time addition of $^{15}NH_4Cl$. ■ control tanks; O ammonium tanks; ● black covered ammonium tanks.

The shrimp muscle amino acids from the black tanks had the highest $\delta^{15}N$ values for all of the amino acids by day 2 when compared to the controls (Figure 5.7). Alanine/threonine (319‰) and proline (307‰) had the largest increases in $\delta^{15}N$ values when day 2 values were compared to day 0 values. The trend in day 8 values was similar to those for the open tanks, with glycine/serine and alanine/threonine having the highest $\delta^{15}N$ values. The slopes of $\delta^{15}N$ versus day for alanine/threonine, arginine, glutamic acid, and valine were statistically higher than those of the controls (slope analysis; overall $p < 0.05$, individual comparisons $p < 0.002$). The limited statistical differences for both open and black covered ammonium tanks in this experiment reflected the large individual differences between shrimp from the same tank. As in OML 99-3, the smallest increase (though not statistically significant) was found for glutamic acid and the largest was for glycine/serine in both open and black tanks.

Contribution of Natural Tank Populations to Shrimp Amino Acid Nitrogen

Estimates of the contribution of tank natural production to shrimp amino acid nitrogen are given in Figure 5.8. In OML 99-3, average percent contribution values ranged from 13.9% in glutamic acid to 55.5% in alanine/threonine. While statistical analysis of the differences between amino acids was not possible due to the limited number of samples, the contributions for glutamic acid and histidine/lysine were

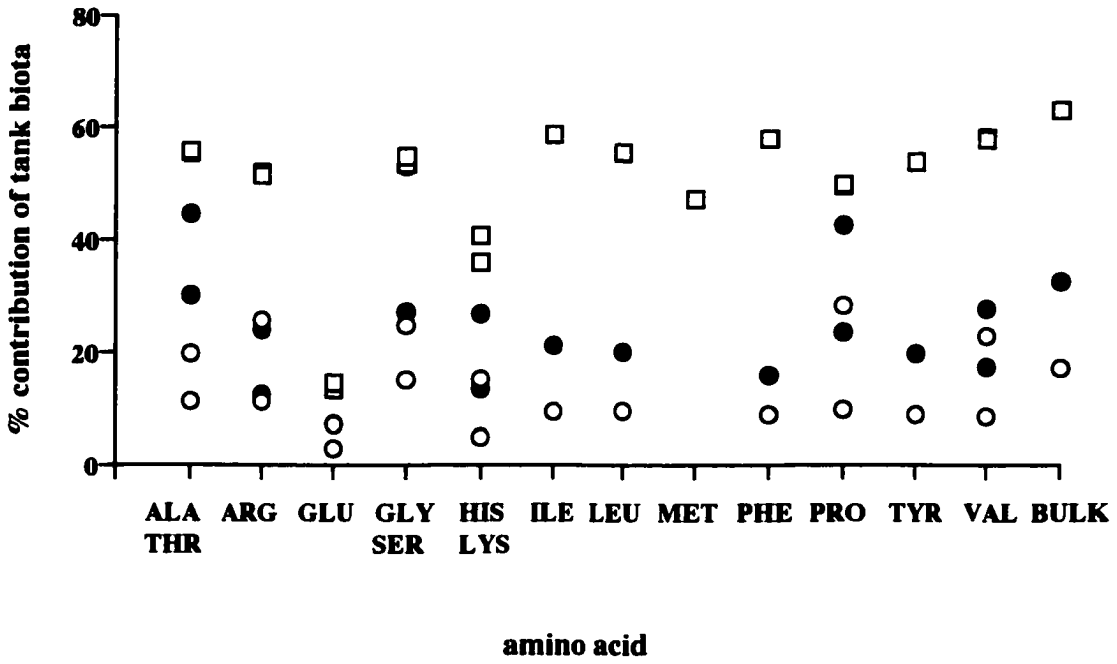

Figure 5.8: Percent contribution of tank biota to shrimp muscle amino acid nitrogen. □ OML 99-3 ammonium tanks (day 11); ○ OML 01-1 ammonium tanks (day 8); ● OML 01-1 black covered ammonium tanks (day 8).

considerably lower than for the other amino acids. The contribution of tank biota to the remaining amino acids in the OML 99-3 trial was similar at approximately 50%. OML 01-1 tank contribution percentages were more variable than in OML 99-3 with the lowest contribution in glutamic acid (5.0% in open tanks and 10.9% in black covered tanks) and highest in glycine/serine (19.9% for open tanks and 40.1% for black). However, the large variation in individual shrimp isotopic values made it difficult to establish a greater contribution for one amino acid over the others or to characterize differences between black covered and uncovered tanks in OML 01-1. GLM analysis indicated that both trial (OML 99-3 ammonium, OML 01-1 ammonium, and OML 01-1 black covered ammonium) and amino acid aid in prediction of the percent contribution ($p < 0.05$). Furthermore, the higher tank biota contribution values found in OML 99-3 versus OML 01-1 were similar to the trends found in the previous chapter for whole muscle tissue (given as 'bulk' in Figure 5.8).

DISCUSSION

The stable isotope data for individual amino acids in shrimp muscle provided strong support for a significant role of tank biota in shrimp nutrition. The estimates of the tank contributions indicated similar results for most of the essential and non-

essential amino acids suggesting that the tank biota provide an important source of amino acids for protein synthesis.

The synthetic pathways for the amino acids in shrimp are generally believed to be similar to those of other animals (Claybrook, 1983) and some of those pathways are indicated in the isotopic results presented here. As noted previously, 10 of the 20 common amino acids found in the shrimp must be provided by the diet. Methionine, tryptophan, and lysine are sometimes questioned as essential amino acids in some species, but this is likely due to their production by associated gut microorganisms (Armitage et al., 1981). Nine of the remaining ten amino acids can be synthesized from glucose. The pathway for asparagine in shrimp, the tenth, is unclear (Dall et al., 1990).

One of the synthesis pathways in crustaceans that varies from other animals is the production of tyrosine from phenylalanine (Claybrook, 1983). This pathway is indicated in this study by the similar increases in ^{15}N for these two amino acids over time. Tyrosine plays a role in the formation of newly molted cuticle (Vacca and Fingerman, 1975) and thus, in these rapidly growing shrimp, would become heavily isotopically labeled. In most organisms, serine is produced from glycolytic intermediates and, in turn, acts as a precursor for glycine (Claybrook, 1983). Radiolabel incorporation experiments in decapods have shown limited labeling of serine relative to

some of the other non-essential amino acids and may indicate low synthesis rates (Claybrook, 1983). Conversely, studies have indicated low labeling of glycine with high serine activity in *Uca pugilator* (Claybrook, 1976) and *Callinectes sapidus* (Gilles and Gerard, 1974). This has led to questions about its biosynthetic pathway. While most of the isotopic information obtained for glycine and serine was as a co-eluted volume, data from individual peaks for these amino acids in OML 99-3 control tanks indicated similar isotopic ratios. These results support serine as a precursor for glycine in the shrimp.

Proline is generally thought to be synthesized from glutamic acid (Claybrook, 1983). However, Dall and Smith (1987) found almost complete depletion of the free amino acid pool of proline during starvation and suggested that *Penaeus esculentus* had limited ability to synthesize this amino acid. Limited incorporation of radiolabel into proline (Claybrook, 1976; van Marrewijk and Zandee, 1975) has also led to the suggestion that it may be synthesized from a compartmentalized precursor pool, specifically arginine (Claybrook, 1983). Minimal labeling of glutamic acid with increased labeling of proline supported the suggestion of an alternate route of synthesis than via glutamic acid. However, it is not clear whether proline in these shrimp was

derived simply by uptake from the tank biota or by combined uptake and production via arginine obtained from the tank biota.

In addition to the more general information indicating the role of tank biota in shrimp amino acid production, the pattern of labeling also indicates potential dietary deficiencies of the formulated feeds. With the assumption that the amino acids of the pond biota were all similarly labeled, all amino acids within the shrimp would be labeled via incorporation of tank biota. Of particular interest, however, was differential labeling potentially due to limiting amounts in the formulated diet. When an animal receives protein in excess of its metabolic requirement, the carbon is respired and the nitrogen lost as ammonia or urea (Dall et al., 1990; Dall and Smith, 1987). Therefore, if the shrimp had a deficiency in a particular amino acid in the feed that was alternately obtained from the tank biota, it was anticipated that it would be labeled to a greater extent than the others. While all the essential amino acids (with the exception of histidine/lysine) were equally labeled within a week after the addition, alanine/threonine exhibited large increases in $\delta^{15}N$ versus control shrimp one day after the addition for each of the trials. The chromatographic co-elution of the non-essential alanine and the essential threonine complicates interpretation of these results. However, alanine, which is obtained from pyruvate, is readily labeled in radiolabel experiments and is likely

produced easily by the shrimp (Claybrook, 1976; Claybrook, 1983). Threonine, on the other hand, must be obtained from the diet and would be labeled as a result of uptake of the labeled tank biota. Although not statistically tested, these results suggest that threonine, an essential amino acid, was less available from the formulated feeds and that the shrimp met their requirements for this amino acid via tank natural production. This may also be the case for methionine, which was rapidly labeled in OML 99-3. However, the limited number of samples obtained for this amino acid and the lack of similar results in OML 01-1 also makes interpretation difficult.

Perhaps more interesting were the amino acids that were only minimally labeled. Glutamic acid and histidine/lysine showed the smallest increases in $\delta^{15}N$ via contribution by tank biota. These were likely the result of an adequate supply of these amino acids from the shrimp feed. Glutamic acid, which was the largest pool of amino acid in the muscle hydrolysates, is readily synthesized from 2-oxoglutarate (Claybrook, 1983). The large size of the pool and its ready synthesis within the shrimp presumably account for the low label incorporation. Histidine and lysine, which are both considered essential for the shrimp, were only slightly labeled in these experiments with contributions by tank biota intermediate between glutamic acid and the other amino acids. Dall and Smith (1987) found lysine to be the second most metabolized amino

acid in *Penaeus esculentus*. However they indicated that this may have reflected excess in their diet. Shrimp in this experiment may have obtained their requirement for these amino acids from their formulated feed, and as a result showed only limited labeling.

The differences in contribution estimates for tank biota to individual amino acids between treatments are not easily explained. As noted in the previous chapter, different growth rates for the shrimp may contribute to differences in the uptake of the label. Growth rates in OML 99-3 were significantly higher than in OML 01-1 and could have, in part, explained the differences in contribution for these two trials. However, growth rates were lower for the black covered tanks than for uncovered ammonium addition tanks in OML 01-1, yet contribution values were generally higher for the black covered tanks. The high values for tanks in which phytoplankton growth was eliminated further suggested a bacterial role in shrimp nutrition. Additionally, the rapid acquisition of this label indicated either direct uptake of ammonium by the shrimp or concentration of the label by bacteria on the feed particles that were then consumed by the shrimp.

CONCLUSIONS

Isotopic labeling of tank biota via pulse ammonium addition indicated variable uptake and assimilation of amino acids by shrimp. Of the essential amino acids, threonine (with alanine) showed large increases in $\delta^{15}N$ for each trial when compared to

control values (17‰ and 29‰ for OML 99-3 and open ammonium tanks in OML 01-1 respectively). Histidine/lysine showed only small increases in $\delta^{15}N$ (2‰ and 7‰ for OML 99-3 and open ammonium tanks in OML 01-1) one day after the addition of the isotope label and limited contribution by tank biota one week after addition. These limited experiments suggested variable requirements by the shrimp for individual amino acids, and that documented pathways of amino acid biosynthesis were consistent with the observed isotopic labeling. The above experiments need to be replicated along with the collection of large enough amounts of tank SPOM to allow characterization of its amino acid isotopic profile in contrast with feed. The information provided by such experiments would be instrumental in further outlining the flow of nitrogen within the food web of cultured shrimp as well as providing more information for optimization of feed for cultured populations. Furthermore, it could provide information that would aid in selection of algal innocula to synthesize the critical balance of amino acids.

CHAPTER 6 – SUMMARY AND SUGGESTED FUTURE STUDIES

This study focused on the development of techniques to analyze nutrient flows in shrimp aquaculture systems through the use of stable isotopic tracers in the diet available to the shrimp. Stable isotopically labeled crystalline amino acids were found to be poor tracers when incorporated into the shrimp's formulated feed, due to dissolution of the label prior to ingestion. Labeled algal cells incorporated into the feeds were much more effective and a much greater proportion of the label was found in shrimp muscle tissue. Concomitant labeling of the tank suspended particulate matter revealed that a significant portion of the label was lost due to inefficient feeding by the shrimp. However, this technique should make it possible to compare different diet formulations and feeding methods.

Labeled algal cells were used to test the effectiveness of stickwater produced in Alaskan pollock fisheries as an attractant/stimulant in shrimp feeds. Both isotopic data and growth data indicated no measurable difference in shrimp growth rates on stickwater amended feeds and feeds with either the standard attractant, squid liver powder, or feeds without an added attractant/stimulant. Further study with the addition of behavioral trials would aid in the interpretation of these results. Additionally, dose/response information for feed with stickwater would be useful given the trend

noted here of increasing percent label incorporation with increasing stickwater concentration.

In addition to examining formulated feed utilization, stable isotope labeling techniques were used to estimate the role of natural tank production in shrimp nutrition via direct labeling of the tank biota. The results indicated that tank biota were significant to shrimp nutrition in these tanks, providing 17 to 77% of shrimp growth nitrogen. One unexpected finding of the examination of the contribution of tank biota to shrimp nitrogen was that shrimp maintained under black covers had similar label uptake as shrimp under open tank conditions, with 23% contribution from the tank. Since the black covers caused an inhibition of algal growth, this experiment may have indicated a larger role of bacteria in shrimp nutrition than previously thought.

The role of tank biota to shrimp nutrition was further examined with a compound specific approach. Individual amino acids from shrimp muscle hydrolysates were monitored for the appearance of isotopic label in tanks in which the label would have been taken up by the biota, and differential labeling of the essential amino acids would indicate any deficiencies in the formulated feed. The limited results suggested that a significant fraction of the essential amino acid requirements was provided by the tank biota. Threonine, in particular, showed a large increase in label one day after the

addition for each trial that could indicate a larger requirement for this essential amino acid over other essentials. Histidine/lysine had a much smaller appearance of the label relative to the other essential amino acids, likely due to sufficient amounts of these amino acids in the feed.

The experiments presented here have provided methods of analysis and highlighted some of the pathways of nutrient flow within the shrimp aquaculture systems. They further indicated several topics that require additional research. The limitation in labeling feed pellets has been dissolution of the label during sloppy feeding by the shrimp. Synthesis of a peptide containing an isotopic label would greatly enhance comparative feed studies due to its high digestibility and low solubility.

The contribution of tank biota to the shrimp is another area that would benefit from more study. While it is clear that the algal communities are providing nutrients to the shrimp, it remains unclear as to the specific nutrients supplied. Component analysis, such as the individual amino acid analysis performed here, would greatly assist in determining the requirements of the shrimp. In addition to further analysis of amino acids profiles, isotopic tracer techniques may provide insights into lipid and carbohydrate requirements of the shrimp.

Additionally, the bacterial role in shrimp nutrition needs reevaluation. While early studies indicated a significant fraction of shrimp nutrition from bacterial sources (Moriarty, 1976; Moriarty, 1977), more recent studies have discounted a large role for bacteria in shrimp growth (Moss and Pruder, 1995; Moss et al., 1992). Because the results presented here were not under normal conditions, it is suggested that labeled mannitol experiments be replicated with a larger stable isotope addition to greatly enrich the SPOM. The increased isotopic enrichment would enhance tracing of any bacterial contribution into shrimp tissues. Double isotopic labeling techniques may also provide a means to separate algal and bacterial contributions to shrimp growth.

In conclusion, the use of stable isotopic labeling techniques in these closed aquaculture systems provides a powerful tool in assessing the nutrient flows under complex conditions. When combined with traditional growth and feed conversion ratio comparisons, stable isotopes may elucidate the ways in which shrimp are dependent on natural production within the tanks. This information, in turn, can be utilized to enhance feeds and limit waste production in these systems.

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APPENDICES

APPENDIX A: Estimates of SPOM carbon and nitrogen for OML trials.

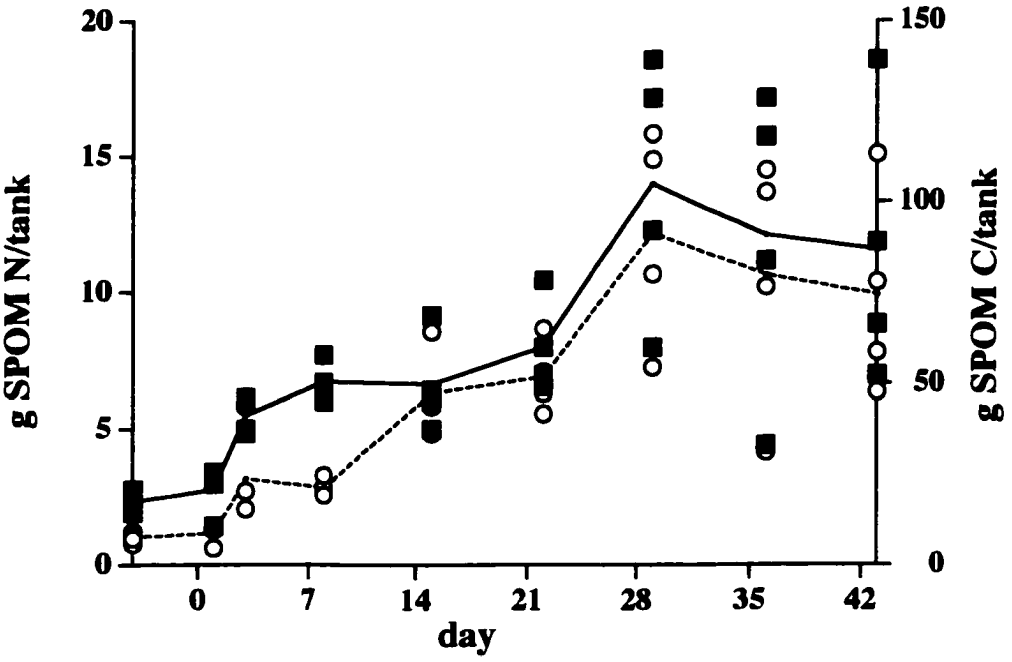

Figure A.1: Changes in SPOM nitrogen and carbon over the length of an experiment for OML 98-1 control tanks. ■ g of SPOM nitrogen/tank; O g of SPOM carbon/tank; solid line = average nitrogen values; dashed line = average carbon values.

Table A.1: Range in SPOM nitrogen and carbon for OML trials. N.D. = not determined.

OML	g SPOM N/tank	g SPOM C/tank
98-1	2.31 – 22.04	4.56 – 164.08
98-5	0.60 – 22.10	4.30 – 120.40
99-3	n.d.	n.d.
01-1	1.00 – 10.20	8.40 - 64.40

APPENDIX B: Comparison of isotopic values for carapace which were scrubbed and unscrubbed using samples from OML 99-3.

Table A.2: $\delta^{15}N$ and $\delta^{13}C$ values for scrubbed and unscrubbed carapace.

Sample ID		Date	$\delta^{15}N$	$\delta^{13}C$
4-12(1)	unscrubbed	5-Aug	75.33	-20.14
	scrubbed		74.29	-20.08
3-11(1)	unscrubbed	5-Aug	54.48	-21.38
	scrubbed		59.47	-20.35
4-6(2)	unscrubbed	12-Aug	5.24	-21.24
	scrubbed		5.38	-20.66
3-3(2)	unscrubbed	12-Aug	8.89	-20.52
	scrubbed		8.92	-20.80
4-11(1)	unscrubbed	19-Aug	4.94	-20.50
	scrubbed		4.75	-20.80

APPENDIX C: Sample calculation for percent of available label in shrimp muscle tissue values.

Step 1: Calculate the atom percent ^{15}N in the feed.

Example:

Tank 1-4 OML 98-5, ^{15}N-glycine addition to the feed.

$$\text{Atom \%} = \frac{100}{\frac{1}{(\delta/1000 + 1) * R_r} + 1}$$

R_r = the ratio of heavy to light isotope for the reference $^{15}N/^{14}N = 0.0036765$

$\delta^{15}N$ for unlabeled feed = 9.05‰

$$\text{Atom \%} = \frac{100}{\frac{1}{(9.05/1000 + 1)\ 0.0036765} + 1}$$

$$= 0.3696$$

$\delta^{15}N$ for labeled feed = 44.86‰

Atom % = 0.3827

Step 2: Determine the atom % excess provided by the labeled feed by subtracting the atom % ^{15}N for the unlabeled feed from the value of the labeled feed.

Excess = 0.3827 – 0.3696 = 0.0131

Step 3: Divide the amount of feed given to a tank by the theoretical number of shrimp in the tank to obtain a value for feed per individual shrimp throughout the experiment.

Day 2 feed = 24.66 g
Shrimp remaining in the tank after sampling = 81
Amount of feed per shrimp = 24.66 g/81 shrimp = 0.30444 g/shrimp

Day 6 feed = 23.75 g
Shrimp remaining in the tank after sampling = 78
Amount of feed per shrimp = 24.66 g/81 shrimp = 0.30449 g/shrimp

Step 4: Calculate the amount of N in the feed. Using mass spectrometry analysis I found that 7.5% of the feed is nitrogen.

Amount N = g feed/shrimp * 0.075
= 0.30444 g/shrimp * 0.075
= 0.022833 g N/shrimp (day 2)

= 0.022837 g N/shrimp (day 6)

Step 5: Calculate the amount of ^{15}N in the feed by multiplying the amount of N by the atom % excess.

Amount ^{15}N = amount N * atom % excess
= 0.022833 g N/shrimp * 0.0131%
= 2.9911×10^{-6} (day 2)

= 0.0228367 g N/shrimp * 0.0131%
= 2.9916 x 10^{-6} (day 6)

Step 6: Calculate the cumulative amount of ^{15}N available to the shrimp over time starting from day 2 (the first full day after label addition).

Cumulative
Day 2 = Day 2 amount ^{15}N (g)
Day 3 = Day 2 + Day 3 amount ^{15}N (g)
Etc.

Day	Amount ^{15}N (g)	Cumulative amount ^{15}N (g)
2	2.9911 x 10^{-6}	2.9911 x 10^{-6}
3	2.9911 x 10^{-6}	5.9822 x 10^{-6}
4	2.9911 x 10^{-6}	8.9734 x 10^{-6}
5	2.9911 x 10^{-6}	1.1964 x 10^{-5}
6	2.9916 x 10^{-6}	1.4956 x 10^{-5}

etc.

Step 7: Calculate atom % ^{15}N for the shrimp muscle tissue.

$\delta^{15}N$ for shrimp muscle, day 6, tank 1-4 = 12.43‰

$$\text{Atom \%} = \frac{100}{\frac{1}{(12.43/1000 + 1)\ 0.0036765} + 1}$$

$$= 0.37084$$

Step 8: Calculate the atom % excess ^{15}N in shrimp muscle by subtracting atom % ^{15}N for control shrimp from atom % ^{15}N for shrimp receiving labeled feed.

Day 6, control tanks $\delta^{15}N = 10.85$
Atom % for control tanks = 0.37026
Atom % excess = 0.37084 – 0.37026 = 0.00058%

Step 9: Calculate the weight of the shrimp using an equation from the linear relationship of day and weight.

Weight = 0.1728*day + 6.5537
Day 6 weight = 7.5905 g

Step 10: Calculate the dry weight of the shrimp. From experimental trials I found that 25% of the wet weight represents the dry weight.

Day 6 dry wt = 7.5905 g * 0.25 = 1.8976 g

Step 11: Calculate the amount of N in the shrimp muscle tissue. From mass spectrometry analysis, I found that 14% of the dry weight is nitrogen.

Day 6 N = 1.8976 g * 0.14 = 0.2657 g

Step 12: Calculate the amount of ^{15}N in the shrimp muscle tissue by multiplying the amount N by the atom % excess.

Amount ^{15}N = amount N * atom % excess
= 0.2657 g N/shrimp * 0.00058% = 1.5409×10^{-6}

Step 13: Determine the percentage of available label present in shrimp muscle by dividing the amount of ^{15}N in shrimp by the cumulative amount of ^{15}N available through the feed and multiplying by 100.

$$\% \text{ label} = \frac{1.5409 \times 10^{-6} \text{ g } ^{15}\text{N shrimp}}{1.4918 \times 10^{-5} \text{ g } ^{15}\text{N feed}} * 100 = 10.33\%$$

APPENDIX D: Feed formulations

Table A.3: Ingredients for reference feeds.

OML 98-1 & OML 98-5		OML 99-3		OML 01-1	
Ingredient	**%**	**Ingredient**	**%**	**Ingredient**	**%**
Fishmeal, menhaden	36.00	Fishmeal - LT 94 (71.83/11.14)	24.50	Fishmeal - LT 94 (76.81/10.71)	11.00
Wheat, whole hard red winter	24.00	Squidmeal - AGX Mexico New (58.94/4.19	2.50	Wheat, whole (15.46/1.71)	47.50
Soybean meal	15.00	Soybean meal (43.84/1.69)	9.50	Vital wheat gluten (80.15/0.94)	4.00
Shrimp meal	10.00	Wheat, whole - HRWW (13.88/1.76)	47.00	Brewers yeast (43.96/0.16)	5.00
Fish solubles, menhaden	8.00	Vital wheat gluten - HFM (72.97/1.06)	4.00	Squid liver powder/Wheat (2.5:7.5)	10.00
Oil, menhaden	2.50	Brewers yeast - Bio-products (40.32/0.29)	3.00	Soybean meal (48.69/1.93)	15.00
Potassium phosphate, dibasic	2.00	Krill meal, hydrolysed (59.38/10.45)	2.00	Soy lecithin - CSM	2.00
Lecithin soybean	1.00	Soy lecithin - CSM	2.00	Fish oil	3.59
Oil, soybean	0.75	Fish oil - Menhaden	3.00	Cholesterol-FG	0.23
Mineral OI mix, P-free with Ca	0.30	Cholesterol - FG	0.23	Potassium phosphate, di (trihydrate)	0.56
Vitamin OI mix	0.30	Potassium phosphate, dibasic	0.56	Sodium phosphate, dibasic	0.56
Stay C-25	0.15	Calcium phosphate, monobasic	0.56	Magnesium phosphate	0.56
		Sodium phosphate, dibasic	0.56		
		AQUAFAN Vitamin Premix - LV99.1	0.40		
		Choline chloride 60%	0.12		
		Stay C-35	0.07		
Total	100	**Total**	100	**Total**	100
Crude Protein (%)	**39.40**	**Crude Protein (%)**	**35.07**	**Crude Protein (%)**	**30.83**
Crude Lipid (%)	**9.20**	**Crude Lipid (%)**	**9.24**	**Crude Lipid (%)**	**8.73**

APPENDIX E: Sample calculation for determining the percent contribution of pond production to shrimp growth nitrogen (tank 1-15, OML 98-1).

The relative contribution of natural production and formulated feed to shrimp growth was determined using the following equation from (Anderson et al., 1987):

$$\frac{W_p}{W_f} = \frac{\delta_t - \delta_g}{\delta_g - \delta_p}$$

The exact steps used in the calculations are as follows:

1. Determine individual shrimp weights using the regression equation from the graph of weight versus day.

   Wt = 0.103*day + 4.288

Day	Weight (g)
1	4.39
8	5.11
15	5.83
22	6.55
29	7.28
36	8.00
43	8.72

2. Multiply wet weight by 0.25 to get dry weight values (dry weight is 25% of wet weight.

Day	Weight (g)	Dry wt (g)
1	4.39	1.10
8	5.11	1.28
15	5.83	1.46
22	6.55	1.64
29	7.28	1.82
36	8.00	2.00
43	8.72	2.18

3. The $\delta^{15}N$ value for the shrimp muscle tissue that is added during the experimental period is calculated using $\delta_g = (W_t\delta_t - W_i\delta_i)/W_g$ where i and t represent the initial value and that at time t and W_g is the weight gained. To account for trophic enrichment from the feed to the shrimp muscle tissue, 3‰ was subtracted from the $\delta^{15}N$ value for muscle tissue (Anderson et al., 1987).

On day 22, δ_g = [(1.64*(51.59-3) – (1.10*9.42)]/0.54
= 128.35‰

Day	Dry wt (g)	Wt gain (g)	Shrimp $\delta^{15}N$	δ_g
1	1.10		9.42	
8	1.28	0.18	42.51	223.39
15	1.46	0.36	47.06	149.90
22	1.64	0.54	51.59	128.38
29	1.82	0.72	57.63	123.70
36	2.00	0.90	49.73	92.33
43	2.18	1.08	62.36	110.22

4. Because the value for the SPOM in the pond is changing throughout the length of the experiment (see graphs with $\delta^{15}N$ values for the NH_4Cl additions), it was necessary to calculate an average $\delta^{15}N$ value for the SPOM.

   - Measured values of SPOM $\delta^{15}N$ were recorded for days -4, 1, 3, 5, 8, 15, 22, 29, 36, and 43
   - Daily values, which weren't sampled directly, were estimated using interpolation.
   - SPOM $\delta^{15}N$ values used in the calculation represented the average SPOM value from day 1 to the day of interest, e.g. $\delta^{15}N$ for day 15 is the average of all values from day 1 to day 15, $\delta^{15}N$ for day 29 is the average of the values from day 1 to day 29.

Day	SPOM $\delta^{15}N$	Ave. $\delta^{15}N$
1	9.40	
8	572.44	573.16
15	278.17	494.35
22	195.73	410.58
29	148.94	352.26
36	113.67	308.81
43	94.35	275.25

5. For the equation from Anderson et al. (1987), the $\delta^{15}N$ for the feed is 9.525‰ (δ_f), the average SPOM is used for δ_p. The ratio of the contribution of tank production (W_p) to added formulated feed (W_f) is:

$$\frac{W_p}{W_f} = \frac{\delta_f - \delta_g}{\delta_g - \delta_p}$$

For day 22,

$$\frac{W_p}{W_f} = \frac{9.525 - 128.38}{128.38 - 410.58}$$

$$= 0.4798$$

Day	W_p/W_f
8	0.61
15	0.41
22	0.42
29	0.50
36	0.38
43	0.61

6. A ratio (W_p/W_f) of 0.42 is 0.42/1.00. Therefore, to calculate the percent contribution by the tank population (W_p), the tank contribution (0.42) is divided by the total contribution by the tank and the feed (0.42 + 1.00). This value is then multiplied by 100.

$$[0.42/(0.42 + 1.00)]*100 = 29.58\%$$

Day	% contribution of tank population
8	37.89
15	29.08
22	29.58
29	33.33
36	27.54
43	37.89

APPENDIX F: Technique for amino acid concentration determination.

Sample preparation

Each sample was prepared to a final volume of 1.2 ml with 50 μl of A and C below and a combination of sample and B to give 1.1 ml (generally 1.05 ml B and 50 μl sample).

A. OPA reagent

20 mg SDS (lauryl sulfate, sodium salt)
10 mg OPA (o-phthaldialdehyde)
1 ml methanol
12 μl 2-mercaptoethanol

B. Borax buffer

22.7 ml 0.1 M NaOH
50 ml 0.025 M borax
27.3 ml DI H_2O
adjust pH to 10.4

C. Internal standard (ISTD) α-aminoadipic acid (0.015 M)

Gradient

A. Na phosphate buffer 0.025M, pH 6.5 + tetrahydrofuran (60 ml in 1940 ml phosphate buffer).

B. Methanol

Gradient profile (1.4 ml/min, 25°C, 340 nm):

		A	B
1.	Initial	85%	15%
2.	0 – 23 min	30%	70%
3.	23 – 28 min	30%	70%
4.	28 – 30 min	85%	15%
5.	30 – 32 min	85%	15%

APPENDIX G: Technique for Alltech Econosphere NH_2 column separation.

Gradient

A. 0.01 M KH_2PO_4, pH 4.3

B. acetonitrile:water (500:70)

Gradient profile (4.0 ml/min, 38°C, 200 nm):

		A	B
1.	Initial	10%	90%
2.	0 – 5 min	10%	90%
3.	5 – 20 min	30%	70%
4.	20 – 27 min	50%	50%
5.	27 – 32 min	10%	90%
6.	32 – 35 min	10%	90%

APPENDIX H: Technique for Alltech Alltima C18 column separation.

Gradient

A. 0.02 M KH_2PO_4, pH 2.5

B. acetonitrile:water (500:70)

Gradient profile (4.0 ml/min, 25°C, 210 nm):

		A	B
1.	Initial	97%	3%
2.	0 – 10 min	97%	3%
3.	10 – 20 min	70%	30%
4.	20 – 21 min	97%	3%
5.	21 – 25 min	97%	3%